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<p>(21) International Application Number: PCT/US92/02050</p> <p>(22) International Filing Date: 12 March 1992 (12.03.92)</p> <p>(30) Priority data:            667,971 12 March 1991 (12.03.91) US            770,967 7 October 1991 (07.10.91) US         </p> <p>(60) Parent Applications or Grants            (63) Related by Continuation            US 667,971 (CIP)            Filed on 12 March 1991 (12.03.91)            US 770,967 (CIP)            Filed on 7 October 1991 (07.10.91)         </p> <p>(71) Applicant (for all designated States except US): BIOGEN, INC. [US/US]; 14 Cambridge Center, Cambridge, MA 02142 (US).</p>		<p>(72) Inventors; and            (75) Inventors/Applicants (for US only) : WALLNER, Barbara, P. [US/US]; 7 Centre Street, Cambridge, MA 02139 (US). MILLER, Glenn, T. [US/US]; 112 Montclair Road, Haverhill, MA 01830 (US). ROSA, Margaret, D. [US/US]; 32 Grove Street, Winchester, MA 01890 (US).</p> <p>(74) Agents: HALEY, James, F., Jr. et al.; Fish &amp; Neave, 875 Third Avenue, 29th Floor, New York, NY 10022-6250 (US).</p> <p>(81) Designated States: AU, CA, JP, US.</p> <p><b>Published</b>  <i>With international search report.            Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p>	

(54) Title: CD2-BINDING DOMAIN OF LYMPHOCYTE FUNCTION ASSOCIATED ANTIGEN 3

## (57) Abstract

Polypeptides and proteins comprising the CD2-binding domain of LFA-3 are disclosed. DNA sequences that code on expression for those polypeptides and proteins, methods of producing and using those polypeptides and proteins, and therapeutic and diagnostic compositions are also disclosed. Deletion mutants unable to bind CD2 and methods for their use are also disclosed. In addition, fusion proteins which comprise the CD2-binding domain of LFA-3 and a portion of a protein other than LFA-3, DNA sequences encoding those fusion proteins, methods for producing those fusion proteins, and uses of those fusion proteins are disclosed.

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CD2-BINDING DOMAIN OF LYMPHOCYTE  
FUNCTION ASSOCIATED ANTIGEN 3

This invention relates to DNA sequences

- 5 encoding and polypeptides which comprise the CD2-binding domain of Lymphocyte Function Associated Antigen 3 ("LFA-3") and to recombinant DNA molecules for expression of those polypeptides. In accordance with this invention, unicellular hosts transformed with
- 10 the DNA sequences and recombinant DNA molecules containing these DNA sequences may be prepared and used to produce proteins and polypeptides comprising the CD2-binding domain of LFA-3. The peptides, polypeptides and proteins of this invention are useful
- 15 in the study of interactions between CD2 and LFA-3, in diagnostic and therapeutic compositions, in antibody screening or purification methods and in other methods of this invention.

BACKGROUND OF THE INVENTION

- 20 T-lymphocytes play a major role in the immune response by interacting with target and antigen presenting cells. T-lymphocyte-mediated killing of target cells is a multi-step process involving adhesion of cytolytic T-lymphocytes to target cells. The
- 25 initiation of the immune response to most antigens involves adhesion of helper T-lymphocytes to antigen-presenting cells.

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These interactions of T-lymphocytes with target and antigen-presenting cells are highly specific and depend on the recognition of an antigen on the target or antigen-presenting cell by one of the many 5 specific antigen receptors on the surface of T-lymphocytes.

The receptor-antigen interaction of T-lymphocytes and other cells is facilitated by various T-lymphocyte surface proteins, e.g., the antigen-10 receptor complex CD3 and accessory molecules CD4, LFA-1, CD8 and CD2. The interaction of T-lymphocytes and other cells is also dependent on accessory molecules, such as ICAM-1, MHC class I and II and LFA-3, that are expressed on the surface of target or 15 antigen-presenting cells and thereby play a role in the action of T-lymphocytes. One general hypothesis is that accessory molecules on the T-lymphocytes and on the target or antigen-presenting cells interact with each other to mediate intercellular adhesion. 20 Accordingly, these accessory molecules are believed to enhance the efficiency of lymphocyte/antigen-presenting cell and lymphocyte/target cell interactions and to be important in cell adhesion-based pathologies (such as leukocyte/endothelial cell interaction leading to 25 pathologic inflammation) and lymphocyte recirculation. Accessory molecules are also involved in activation of lymphocytes.

One important example of cell-cell interaction mediated by accessory molecules is the 30 specific interaction between CD2 (a T-lymphocyte accessory molecule) and LFA-3 (a target cell accessory molecule). CD2/LFA-3 binding appears to be essential for many important cell-cell reactions, including the initiation of the T-lymphocyte functional responses 35 (Dustin et al., J. Exp. Med., 165, pp. 677-92 (1987);

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Springer et al., Ann. Rev. Immunol., 5, pp. 223-52 (1987)). The importance of the CD2/LFA-3 complex in cell-cell adhesion is indicated by the findings that purified LFA-3 binds to CD2 on the surface of

5 T-lymphocytes (Dustin et al., J. Exp. Med., 165, pp. 677-92 (1987)), that CD2 purified from T-lymphocytes binds LFA-3 on cell surfaces and inhibits the binding of LFA-3-specific monoclonal antibodies ("MAbs") to LFA-3 (Selvaraj et al., Nature, 326,

10 pp. 400-403 (1987)), and that rosetting of human erythrocytes, which express LFA-3, to cells expressing CD2 is blocked by anti-LFA-3 MAbs and anti-CD2 MAbs (see, e.g., Seed et al., Proc. Natl. Acad. Sci. USA, 84, pp. 3365-69 (1987)).

15 LFA-3, which is found on the surface of a wide variety of cells including monocytes, granulocytes, T-lymphocytes, erythrocytes, B-lymphoblastoid cell lines, thymic epithelial cells, and vascular endothelial cells, has become the subject

20 of a considerable amount of study to further elucidate its role in various T-lymphocyte interactions. Two natural forms of LFA-3 have been identified. One form of LFA-3 ("transmembrane LFA-3") is anchored in the cell membrane by a transmembrane hydrophobic domain.

25 cDNA encoding this form of LFA-3 has been cloned and sequenced (see, e.g., Wallner et al., J. Exp. Med., 166, pp. 923-32 (1987)). Another form of LFA-3 is anchored to the cell membrane via a covalent linkage to phosphatidylinositol ("PI")-containing glycolipid.

30 This latter form has been designated "PI-linked LFA-3", and cDNA encoding this form of LFA-3 has also been cloned and sequenced (Wallner et al., PCT patent application WO 90/02181).

Although the DNA sequence of the LFA-3 gene  
35 and the primary amino acid sequences of both forms of

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LFA-3 have been determined, the actual site of interaction between LFA-3 and its receptor, CD2, has not previously been identified. There is a need to identify the CD2-binding domain on LFA-3 in order to 5 better understand the specific interaction between CD2 and LFA-3 and, thereby, to effect and modulate the cellular and immunological processes that are dependent on the formation of the CD2/LFA-3 complex. Such information would also be useful in a variety of other 10 applications including diagnostic and therapeutic compositions, protein purification, antibody identification and purification, and comparative and structural studies of LFA-3 and other proteins.

SUMMARY OF THE INVENTION

15 This invention addresses the aforementioned needs by identifying the CD2-binding domain of LFA-3 and providing nucleotide sequences defining the CD2-binding region of LFA-3 and CD2 binding polypeptides encoded by those sequences. The present invention 20 provides polypeptides having the amino acid sequence: X<sub>1</sub>-X<sub>2</sub>-(SEQ ID NO:1) Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:

X<sub>1</sub> is hydrogen or methionyl;

X<sub>2</sub>, if present, is a polypeptide having the

25 following amino acid sequence or a portion thereof consisting of the carboxy terminal 1 to 77 amino acids of the sequence (SEQ ID NO:5): Val Ala Gly Ser Asp Ala Gly Arg Ala Leu Gly Val Leu Ser Val Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe Ser Gln Gln Ile Tyr Gly 30 Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

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Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino-terminal 1 to 32 amino acids of the sequence (SEQ ID NO:33): Ser Leu Thr Ile Tyr Asn Leu Thr Ser 5 Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser Pro Asn Ile Thr Asp Thr Met Lys Phe Phe Leu Tyr Val;  
and analogs and derivatives thereof,  
said polypeptides being capable of binding to CD2.

In another embodiment, the present invention 10 provides polypeptides having the amino acid sequence:  
 $X_1-X_2-(\text{SEQ ID NO:1})$  Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:

$X_1$  is hydrogen or methionyl;  
 $X_2$ , if present, is a polypeptide having the 15 following amino acid sequence or a portion thereof consisting of the carboxy-terminal 1 to 50 amino acids of the sequence (SEQ ID NO:2): Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys 20 Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino-terminal 1 to 10 amino acids of the sequence 25 (SEQ ID NO:3): Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser;

and analogs and derivatives thereof,  
said polypeptides being capable of binding to CD2.

The invention also provides DNA sequences 30 encoding the above polypeptides, recombinant molecules containing those DNA sequences, hosts transfected with such DNA sequences and molecules and methods of making the polypeptides recombinantly, synthetically or semi-synthetically.

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The polypeptides of this invention are useful in diagnosing diseases characterized by the presence or absence of CD2 on the surface of particular cells and to treat pathologies dependent on the formation of the  
5 LFA-3/CD2 complex.

The polypeptides of this invention and the DNA sequences encoding them may also be used to prepare recombinant or synthetic fusion proteins, which comprise a functional CD2-binding domain or polypeptide  
10 of LFA-3, as defined above, and another domain of a protein or polypeptide other than LFA-3. The CD2-binding domain portion of the fusion proteins allows the other polypeptides to be targeted specifically to CD2-expressing cells (CD2<sup>+</sup> cells). DNA sequences  
15 encoding these fusion proteins are also part of this invention.

One example of such fusion proteins of this invention is novel fusion proteins containing a portion of LFA-3 containing a functional CD2-binding domain, as  
20 defined above, fused to at least a portion of the Fc region of an immunoglobulin (Ig). Unexpectedly, the LFA-3-Ig fusion proteins of this invention inhibit T-lymphocyte activation and proliferation of peripheral blood lymphocytes.

25 It is a further aspect of this invention that the polypeptides and fusion proteins of this invention may also be used to label CD2 molecules, for example, soluble CD2, in solution or on the surface of T-lymphocytes or other CD2-expressing cells. The  
30 polypeptides and fusion proteins of this invention may also be used to label any protein, polypeptide or peptide comprising the domain of CD2 which binds LFA-3, for example, in a fusion protein generated using recombinant DNA techniques.

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The polypeptides and fusion proteins of this invention may also be used in affinity chromatography to purify CD2 or any other polypeptide or protein conjugate containing the domain of CD2 which binds

5 LFA-3.

This invention also provides altered (mutant) forms of the LFA-3 protein which lack the CD2-binding domain and the DNA sequences encoding such "deletion mutant" forms of LFA-3. Mutant proteins, which lack  
10 the CD2-binding domain of LFA-3, as defined above, may be used *in vivo* or *in vitro* to generate or to bind antibodies or other molecules that recognize epitopes or sites on LFA-3 other than the CD2-binding domain.

In addition to the monomeric form of the  
15 CD2-binding polypeptides and fusion proteins of this invention, multimeric forms are also enabled by this invention. Such forms may have enhanced affinity for CD2, enhanced immunogenicity and/or enhanced ability to initiate T-lymphocyte functional responses, e.g.,  
20 stimulation of T-cell activation, through more effective or multiplied formation of CD2/LFA-3 complexes. Also, such multimeric forms may be more effective in competitive binding of CD2, making them more useful as immunosuppressants and more sensitive as  
25 diagnostics or reagents.

In addition, this invention contemplates antibodies recognizing the polypeptides and fusion proteins of this invention. Polyclonal and monoclonal antibodies to the polypeptides and fusion proteins of  
30 this invention may be obtained by immunizing an animal with polypeptides or fusion proteins of this invention.

Low molecular weight (generally less than 1000 daltons) inhibitors of the formation of the CD2/LFA-3 complex are also provided by this invention.  
35 Such "small molecule" inhibitors may be produced in

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vitro by synthetic methods and may comprise part of the amino acid sequences of the polypeptides of this invention or may be entirely non-peptidyl organic molecules having a structural conformation that is able

5 to mimic the binding specificity of the polypeptides and fusion proteins of this invention to CD2.

This invention also provides methods, compositions and kits for diagnostic and therapeutic uses in which the presence or absence of the

10 CD2-binding domain of LFA-3 is desirable.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 (Fig. 1A and Fig. 1B taken together) depicts amino acid (SEQ ID NO:10) and nucleotide (SEQ ID NO:9) sequences of transmembrane LFA-3 indicating

15 (with underlining) deletions made in the extracellular region of LFA-3. Regions designated M53, M54, M55, M56, M57, M58, M59, M60, M61, M62, M63, M64, M65, M66, M90, M91 and M92, respectively, were looped out to create recombinant genes encoding 17 separate deletion

20 mutants. Expression of the deletion mutants in mammalian cells provided altered LFA-3 surface proteins missing a segment of amino acids present in the native LFA-3 protein. For example, expression of the deletion mutant designated M57 (that is, the LFA-3 DNA of Fig. 1

25 having the M57 region deleted) in CHO cells resulted in a mutant LFA-3 that did not bind to CD2. The amino terminal methionine (Met) is designated -28 to indicate that it is the amino terminal residue of the 28-amino acid signal peptide sequence of preLFA-3. The amino

30 terminal residue of mature LFA-3 is a phenylalanine (Phe) and is designated +1.

Figure 2 (Figs. 2A, 2B, 2C, 2D, 2E, 2F, 2G, 2H, 2I, 2J, 2K, 2L, 2M and 2N taken together) depicts results of immunofluorescence flow cytometry by FACS

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analysis of transfected CHO cells expressing mutant forms of LFA-3 encoded by deletion mutants M57 (Figs. 2A and 2B), M65 (Figs. 2C and 2D), M63 (Figs. 2E and 2F), M54 (Figs. 2G and 2H), M55 (Figs. 2I and 2J),

5 M56 (Figs. 2K and 2L), and M58 (Figs. 2M and 2N).

Transfected cells were reacted with anti-LFA-3 polyclonal antiserum 202 (dashed lines, Figs. 2A, 2C, 2E, 2G, 2I, 2K, 2M), anti-LFA-3 MAb TS2/9 (dashed lines, Figs. 2B, 2D, 2F, 2H, 2J, 2L, 2N), or anti-LFA-3

10 MAb 7A6 (dotted lines, Figs. 2B, 2D, 2F, 2H, 2J, 2L, 2N). In each analysis, the control peak (solid line) represents the cell population with background levels of fluorescein isothiocyanate-conjugated (FITC) goat anti-mouse or goat anti-rabbit IgG binding.

15 Figure 3 (Figs. 3A, 3B and 3C taken together) depicts the results of a Jurkat cell binding experiment. The photomicrographs show Jurkat cells expressing CD2 mixed with normal CHO cells (negative control) (Fig. 3A), M57/CHO cells (Fig. 3B) and P24/CHO 20 cells expressing PI-linked LFA-3 (positive control) (Fig. 3C).

Figure 4 depicts a Northern gel analysis of LFA-3 gene transcripts synthesized in M57/CHO cells. Filters containing mRNA samples from CHO negative 25 control cells (lane 1), M57/CHO cells (lane 2) and M16.3/CHO positive control cells (lane 3) were probed with a NotI restriction fragment of plasmid pLFA3M54, containing nucleotides 1-153 and 184-1040 of the LFA-3 cDNA sequence (see Figure 1), labeled with <sup>32</sup>P by nick- 30 translation.

Figure 5 shows an autoradiogram depicting the differential immunoprecipitation of proteins from <sup>125</sup>I surface-labeled M57/CHO cells (lanes 1-4), normal CHO cells (negative control) (lanes 5-7), and P24/CHO cells 35 (positive control) (lanes 8-11). Lysates of surface-

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labeled cells were reacted with anti-LFA-3 MAb TS2/9 (lanes 2, 5, 9), anti-LFA-3 MAb 7A6 (lanes 3, 6, 10), or anti-LFA-3 polyclonal antiserum 202 (lanes 4, 7, 11). The antibody-precipitated proteins were 5 electrophoresed on SDS-polyacrylamide gels prior to autoradiography.

Figure 6 (Figs. 6A and 6B taken together) depicts amino acid (SEQ ID NO:10) and nucleotide (SEQ ID NO:9) sequences of transmembrane LFA-3 indicating 10 (with underlining) deletions in the extracellular region of LFA-3. Regions designated M100, M101, and M102, respectively, were looped out to create recombinant genes encoding 3 separate deletion mutants.

Figure 7 (Figs. 7A, 7B, 7C and 7D taken 15 together) shows results of Jurkat cell-binding experiments in which Jurkat cells expressing CD2 were mixed with (a) normal CHO cells, (b) M100/CHO cells, (c) M101/CHO cells, or (d) M102/CHO cells.

Figure 8 (Figs. 8A, 8B and 8C taken together) 20 shows Jurkat cell binding by LF08/AffiGel-10 beads (see Example 8, *infra*). LF08/AffiGel-10 beads were mixed with Jurkat cells and the cell-bead binding observed under a microscope (Figs. 8A and 8B). For comparison (negative control), a non-LFA-3 peptide from hepatitis 25 B (i.e., "MXC 01", (SEQ ID NO:4) Thr Lys Pro Asp Leu Val Asp Lys Gly Thr Glu Asp Lys Val Val Asp Val Val Arg Asn) was fixed to Affigel-10 beads, the beads mixed with Jurkat cells, and cell-bead binding recorded in Fig. 8C.

Figure 9 depicts the amino acid (SEQ ID 30 NO:12) and nucleotide (SEQ ID NO:11) sequences of PI-linked LFA-3 indicating (with underlining) the internal deletion made in the nucleotide sequence and corresponding mutant PI-linked LFA-3 protein. The 35 region of the DNA designated PIM3 was looped out to

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create a recombinant gene encoding a mutant protein containing the N-terminal 89 amino acids of PI-linked LFA-3 but lacking the subsequent 71 amino acids.

5 Expression of the deletion mutant PIM3 (that is, the PI-linked LFA-3 DNA of Fig. 9 having the PIM3 region deleted) in CHO cells resulted in a mutant form of PI-linked LFA-3 that retained a CD2-binding domain.

Figure 10 (Figs. 10A and 10B taken together) depicts results of immunofluorescence flow cytometry by 10 FACS analysis of transfected CHO cells expressing a mutant form of PI-linked LFA-3 encoded by deletion mutation PIM3. Transfected cells (PIM3.25.2 cells) expressed a surface protein that was recognized by anti-LFA-3 MAb TS2/9 (Figure 10A, dashed line) and that 15 was susceptible to release from the cell surface by PI-PLC treatment (Figure 10A, dotted line). Figure 10B depicts the results of a similar analysis using transfected CHO cells amplified for expression of the mutant PI-linked protein (PIM2.25.2.100.12 cells). 20 These cells produced a protein on their cell surface recognized by MAb 7A6 (Figure 10B, dashed line) and that was susceptible to release from the cell surface by PI-PLC treatment (Figure 10B, dotted line). In each analysis, the control peak (solid line) represents the 25 cell population with background levels of FITC-conjugated goat anti-mouse IgG binding.

Figure 11 is a diagram of a dimer of LFA3TIP, depicting the various domains of this LFA-3-Ig fusion protein. As depicted in this figure, "LFA-3" refers to 30 the amino terminal 92 amino acids of mature LFA-3. "H" refers to the ten amino acids of the IgG1 hinge region containing two cysteines to form two intermolecular disulfide bonds. Each disulfide bond is depicted as horizontal double SS. "C<sub>H</sub>2" and "C<sub>H</sub>3" refer to the two

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constant domains present in the Fc region, below the hinge region, in human IgG1 molecules.

Figure 12 depicts the amino acid (SEQ ID NO:43) and nucleotide (SEQ ID NO:42) sequences of 5 preLFA3TIP (i.e., LFA-3 (amino acids +1-+92) and the 28-amino acid signal sequence) and the various domains of the fusion protein. The nucleotide sequence of Figure 12 is also the same as the DNA sequence insert in the expression plasmid pSAB152.

10 Figure 13 is a photograph of a Western blot analysis of SDS-PAGE gels under nonreducing (lanes 1 and 2) and reducing (lanes 3 and 4) conditions. Lanes 1 and 3 are high molecular weight markers (BRL, Gaithersburg, Maryland). Lanes 2 and 4 contain LFA3TIP 15 purified as described in Example 13.

Figure 14 (Figs. 14A and 14B taken together) depicts results of immunofluorescence flow cytometry by FACS analysis of Jurkat cells incubated with LFA3TIP plus R-Phycoerythrin conjugated anti-human IgG F(ab')<sub>2</sub> 20 (dotted lines) in the presence of anti-CD2 MAb TS2/18 plus FITC conjugated goat anti-mouse IgG(H+L) F(ab')<sub>2</sub> (small dots in Fig. 14A) or anti-CD2 ascites fluid T11<sub>1</sub> (dashes in Fig. 14A), T11<sub>2</sub> (dashes in Fig. 14B) or T11<sub>3</sub> (small dots in Fig. 14B), plus FITC conjugated goat 25 anti-mouse IgG(H+L) F(ab')<sub>2</sub>.

Figure 15 depicts the results of a human allogeneic mixed lymphocyte reaction (MLR) assay for T-cell activation as measured by <sup>3</sup>H-thymidine incorporation into T-cells in the presence of anti- 30 LFA-3 MAb 1E6 (closed squares), LFA3TIP (closed circles) and nonspecific human IgG1 (closed triangles).

Figure 16 depicts the results of MLR assays for inhibition of T-cell activation by selected proteins. "7A6" and "1E6" are anti-LFA-3 monoclonal 35 antibodies (MAbs), specific for the CD2-binding domain

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of LFA-3. "TS2/18" is an anti-CD2 MAb. "LFA3IgGA" and "LFA3IgG72A" are preparations of LFA3TIP differing in purity, i.e., 75% and 50%, respectively. hIgG is nonspecific human IgG. "PI-LFA3" is multimeric PI-linked LFA-3. "CD4-IgG" is a fusion protein consisting of a portion of the CD4 protein fused to a portion of the Fc region of IgG. "Mock" refers to a "mock preparation" purified from COS7 cells transfected with an expression vector pSAB132 lacking the DNA sequence encoding LFA3TIP.

Figure 17 depicts results of a PBL proliferation assay as measured by incorporation of  $^3\text{H}$ -thymidine. PBL proliferation was measured in the presence of LFA3TIP (closed squares), human IgG (closed circles) and a "mock preparation" containing the contaminant ("contamination") which co-purifies with the LFA3TIP used in this assay (closed triangles).

Figure 18 depicts results of an OKT3 (anti-CD3 MAb) dependent PBL proliferation assay as measured by incorporation of  $^3\text{H}$ -thymidine. PBL proliferation was measured in the presence of 3 nM OKT3 (OKT3), 3 nM OKT3 plus 1 nM or 10 nM LFA3TIP (OKT3 + LFA3TIP), 3 nM OKT3 plus 1 nM or 10 nM human IgG1 (OKT3 + hIgG1), or medium with no OKT3 present (medium).

Figure 19 depicts results of a phytohemagglutinin (PHA) dependent PBL proliferation assay as measured at a suboptimal stimulatory concentration (0.1  $\mu\text{g}/\text{ml}$ ) and at an optimal stimulatory concentration (1  $\mu\text{g}/\text{ml}$ ) of PHA. PBL proliferation was measured as indicated in the presence of PHA alone (PHA) and in the presence of PHA plus: PI-linked LFA-3 (+PILFA-3); monomeric soluble LFA-3 (+mon LFA-3); LFA3TIP (+LFA3TIP); an LFA-3-IgG fusion protein which lacks the 10 amino acid M57 region of LFA-3 involved in CD2 binding (+M57IgG); anti-LFA-3 1E6 MAb (+1E6); anti-

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CD2 TS2/18 MAb (+TS2/18); and a full-length LFA-3-Ig fusion protein (+FLIgG). Each of the added molecules was present at 5 µg/ml.

DETAILED DESCRIPTION OF THE INVENTION

5       The polypeptides, compositions, and methods of the present invention are characterized by polypeptides having the amino acid sequence:  $X_1-X_2-$  (SEQ ID NO:1)  
Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:

10       $X_1$  is hydrogen or methionyl;  
 $X_2$ , if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy-terminal 1 to 77 amino acids of the sequence (SEQ ID NO:5): Val Ala Gly Ser Asp Ala  
15      Gly Arg Ala Leu Gly Val Leu Ser Val Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe  
20      Lys;

Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino-terminal 1 to 32 amino acids of the sequence (SEQ ID NO:33): Ser Leu Thr Ile Tyr Asn Leu Thr Ser  
25      Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser Pro Asn Ile Thr Asp Thr Met Lys Phe Phe Leu Tyr Val;  
and analogs and derivatives thereof,  
said polypeptides being capable of binding to CD2.

In another embodiment, the polypeptides of  
30     this invention have the amino acid sequence:  $X_1-X_2-$  (SEQ ID NO:1) Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:  
 $X_1$  is hydrogen or methionyl;

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$X_2$ , if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy-terminal 1 to 50 amino acids of the sequence (SEQ ID NO:2): Phe Ser Gln Gln Ile Tyr  
5 Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

Y is hydroxyl or a polypeptide of the following  
10 amino acid sequence or a portion thereof consisting of the amino-terminal 1 to 10 amino acids of the sequence (SEQ ID NO:3): Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser;

and analogs and derivatives thereof,  
15 said polypeptides being capable of binding to CD2.

Preferably, the polypeptides of this invention have the amino acid sequence of amino acids 29-120 of SEQ ID NO:10, amino acids 29-108 of SEQ ID NO:10, amino acids 48-108 of SEQ ID NO:10, and SEQ ID  
20 NO:7.

Throughout this specification and in the claims, the abbreviations employed for amino acids and their residues are used in conformity with the generally accepted rules of nomenclature and relate to  
25  $\alpha$ -amino acids and their residues of the L-series.

A derivatized amino acid is a natural or non-natural amino acid in which the normally occurring side chain or end group is modified by chemical reaction. Such modifications include, for example, gamma-  
30 carboxylation,  $\beta$ -hydroxylation, sulfation, sulfonation, phosphorylation, amidization, esterification, t-butoxy carbonylation, N-acetylation, carbobenzoylation, tosylation, benzylation, and other modifications known in the art.

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A derivatized polypeptide is a polypeptide containing one or more derivatized amino acids.

Analogs of the polypeptides of this invention may be characterized, for example, by amino acid

- 5 substitutions, additions or deletions, or utilization of D-amino acids. The preferred substitutions in the polypeptides of this invention are those that are recognized in the art to be conservative amino acid substitutions. For example, amino acids belonging to
- 10 one of the following groups represent conservative changes: ala, pro, gly, glu, asp, gln, asn, ser, thr; cys, ser, tyr, thr; val, ile, leu, met, ala, phe; lys, arg, his; and phe, tyr, trp, his. See, e.g., Grantham, Science, 185, pp. 862-64 (1974); Dayhoff, In Atlas of
- 15 Protein Sequence and Structure, 5, 1978; Argos, EMBO J., 8, pp. 779-785 (1989).

It should be understood that all analogues and derivatives of this invention are characterized by biological activities that are similar to those of the

- 20 CD2-binding polypeptides described herein.

Accordingly, these analogs and derivatives may be employed in compositions, combinations and methods for diagnosis, therapy and prophylaxis in the same manner as the polypeptides of this invention.

- 25 The production of the polypeptides of this invention may be achieved by a variety of methods known in the art. For example, the polypeptides may be derived from the intact transmembrane or PI-linked LFA-3 molecules by proteolysis using specific
- 30 endopeptidases in combination with exopeptidases, Edman degradation, or both. The intact LFA-3 molecules, in turn, may be purified from natural sources using conventional methods. Alternatively, full-length LFA-3 or truncated forms of LFA-3 may be produced by known

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recombinant DNA techniques using cDNAs. (See, United States patent 4,956,281 to Wallner et al.)

Preferably, the polypeptides of the present invention are produced directly, thus eliminating the  
5 need for a larger LFA-3 as a starting material. This may be achieved by conventional chemical peptide synthesis techniques or by well-known recombinant DNA techniques, wherein only those DNA sequences which encode the desired polypeptides are expressed in  
10 transformed hosts.

A gene which encodes the desired LFA-3 polypeptide of this invention may be designed based on the amino acid sequence of the desired polypeptide.

Standard methods may be then applied to synthesize the  
15 gene. For example, the amino acid sequence may be used to construct a back-translated gene. A DNA oligomer containing a nucleotide sequence capable of coding for an LFA-3 polypeptide of this invention may be synthesized in a single step. Alternatively, several  
20 smaller oligonucleotides coding for portions of the desired polypeptide may be synthesized and then ligated together. Preferably, the DNA sequence encoding an LFA-3 polypeptide of this invention will be synthesized as several separate oligonucleotides which are  
25 subsequently linked together. The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

Once assembled, preferred genes will be characterized by sequences that are recognized by  
30 restriction endonucleases (including unique restriction sites for direct assembly into a cloning or an expression vector), preferred codons taking into consideration the host expression system to be used, and a sequence which, when transcribed, produces a  
35 stable, efficiently translated RNA. Proper assembly

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may be confirmed by nucleotide sequencing, restriction mapping, and expression of a biologically active polypeptide in a suitable host.

One embodiment of a DNA sequence according to  
5 this invention comprises the nucleic acid sequence  
encoding amino acids 1 through 70 of mature LFA-3,  
i.e., nucleotides 94 through 303 in Figure 1. Another  
preferred embodiment comprises the nucleic acid  
sequence (SEQ ID NO:6): AATAGGGTTT ATTTAGACAC  
10 TGTGTCAGGT, which codes for amino acids 51-60 of mature  
LFA-3. However, the preferred DNA sequences of this  
invention encode polypeptides having the amino acid  
sequences of amino acids 29-120 of SEQ ID NO:10, amino  
acids 29-108 of SEQ ID NO:10, amino acids 48-108 of  
15 SEQ ID NO:10, and SEQ ID NO:7. The most preferred DNA  
sequence of this invention encodes a polypeptide having  
the amino acid sequence 1-120 of SEQ ID NO:10. This  
sequence when employed in animal cells allows  
production, secretion and maturation of a polypeptide  
20 having the amino acid sequence of amino acids 29-120 of  
SEQ ID NO:10.

It will be appreciated by those of skill in  
the art that, due to the degeneracy of the genetic  
code, DNA molecules comprising many other nucleotide  
25 sequences will also be capable of encoding the  
polypeptides of this invention. These degenerate  
sequences are encompassed by the present invention.

The present invention also relates to  
recombinant DNA molecules comprising the aforementioned  
30 DNA sequences. The recombinant DNA molecules of this  
invention are capable of directing expression of the  
LFA-3 polypeptides of this invention in hosts  
transformed therewith. A DNA sequence encoding an  
LFA-3 polypeptide of this invention must be operatively  
35 linked to an expression control sequence for such

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expression. The term "operatively linked" as used herein refers to positioning in a vector such that transcription and translation of the coding sequence is directed by the control sequence.

- 5 To construct a recombinant DNA molecule capable of directing expression of the LFA-3 polypeptides of this invention, the DNA sequences encoding these polypeptides may be inserted into and expressed using a wide variety of vectors.
- 10 Furthermore, within each specific expression vector, various sites may be selected for insertion of these DNA sequences. These sites are usually designated by the restriction endonuclease which cuts them. They are well recognized by those of skill in the art. It will
- 15 be appreciated, however, that an expression vector useful in this invention need not have a restriction endonuclease site for insertion of the chosen DNA fragment. Instead, the vector may be joined to the fragment by alternative means.
- 20 The expression vector, and in particular the site chosen for insertion of a selected DNA fragment and operative linking to an expression control sequence, is determined by a variety of factors. These factors include, e.g., the number of sites susceptible
- 25 to a particular restriction enzyme, the size of the polypeptide to be expressed, susceptibility of the desired polypeptide to proteolytic degradation by host cell enzymes, contamination or binding of the polypeptide to be expressed by host cell proteins
- 30 difficult to remove during purification, expression characteristics, such as the location of start and stop codons relative to the vector sequences, and other factors recognized by those skilled in the art. The choice of a vector and an insertion site for a DNA
- 35 sequence is determined by a balance of these factors

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and not all selections will be equally effective for a given case.

Useful expression vectors for eukaryotic hosts include, for example, vectors comprising

- 5 expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus, and vectors useful specifically in insect cells, such as pVL 941. Useful bacterial expression vectors include known bacterial plasmids, e.g., plasmids from E.coli
- 10 including colE1, pCR1, pBR322, pMB9 and their derivatives; wider host range plasmids, such as RP4; the numerous derivatives of phage lambda, e.g., NM 989 and the lambda gt series; other DNA phages, e.g., M13 and other filamentous single-stranded DNA phages; and
- 15 commercially available high expression vectors, e.g., the pGEM series and the lambda Zap vectors. Useful mammalian cell expression vectors include, for example, pNUT. Vectors useful in yeasts include, for example, the 2 $\mu$  plasmid and derivatives thereof.

- 20 Such expression vectors are also characterized by at least one expression control sequence that may be operatively linked to the DNA sequences of this invention inserted in the vector in order to control and to regulate the expression of that
- 25 cloned DNA sequence. Examples of useful expression control sequences include the malE system, the OmpA system, the lac system, the trp system, the tac system, the trc system, major operator and promoter regions of phage lambda, the control region of fd coat protein,
- 30 the glycolytic promoters of yeast, e.g., the yeast acid phosphatase, e.g., Pho5, the promoters of the yeast mating factors, and promoters derived from polyoma, adenovirus, retrovirus, and simian virus, e.g., the early and late promoters of SV40, eukaryotic cell
- 35 promoters, such as the metallothionein promoter and

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other sequences known to control the expression of genes of prokaryotic or eukaryotic cells and their viruses or combinations thereof.

The recombinant DNA molecules of the present invention may also comprise other DNA coding sequences fused to and in frame with the DNA sequences of this invention. For example, such constructs may be characterized by an ATG start codon fused directly to the nucleotides encoding the first amino acid of the LFA-3 polypeptide. This construction may produce an f-Met polypeptide. However, it will be understood that the initial methionine may be cleaved during expression in a transformed host or may be subsequently removed. Alternatively, a DNA sequence encoding a bacterial or eukaryotic signal sequence may be fused to the 5' end of a DNA sequence encoding an LFA-3 polypeptide of this invention. This would allow the expressed product to be either secreted or targeted to a specific subcellular compartment within the host cell. Most signal sequences are removed by the host cell after performing their targeting function, thus obviating the need for removal after purification of the desired polypeptide. Many signal sequences, as well as the DNA sequences encoding them, are known in the art. The fusion protein of such signal sequence DNA to and in frame with the sequence encoding an LFA-3 polypeptide of this invention can be achieved by standard molecular biology techniques.

Alternatively, a DNA sequence encoding an LFA-3 polypeptide of this invention may be expressed as a fusion protein by in-frame ligation to a second DNA sequence encoding a host cell polypeptide. The expression of a fusion protein may afford several advantages, such as increased resistance to host cell degradation, ease of identification based upon the

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activity or antigenicity of the host cell polypeptide, and ease of purification, based upon the physical or immunological properties of the host cell polypeptide.

This invention also relates to hosts

5 transformed with the recombinant DNA molecules described above. Useful hosts which may be transformed with these recombinant DNA molecules and which may be employed to express the LFA-3 polypeptides of this invention may include well known eukaryotic and  
10 prokaryotic hosts, such as strains of E.coli, e.g., E.coli SG-936, E.coli HB 101, E.coli W3110, E.coli X1776, E.coli X2282, E.coli DH1, E.coli DH5-alpha, E.coli MRC1; strains of Pseudomonas; strains of Bacillus, such as Bacillus subtilis; strains of  
15 Streptomyces; strains of Saccharomyces; animal cells, such as COS cells, CHO cells, BHK cells, human tissue cells; insect cells (e.g., Spodoptera frugiperda (SF9)); and plant cells in tissue culture. The preferred host for polypeptides claimed herein is CHO  
20 cells.

It will be appreciated that not all host/expression vector combinations will function with equal efficiency in expressing DNA sequences encoding the LFA-3 polypeptides of this invention. However, a  
25 particular selection of a host-expression vector combination may be made by those of skill in the art after due consideration of the principles set forth herein without departing from the scope of this invention. For example, the selection should be based  
30 on a balancing of a number of factors. These factors include, for example, compatibility of the host and vector, toxicity of the polypeptides encoded by the DNA sequence to the host, vector copy number and the ability to control that copy number, the expression of  
35 other proteins encoded by the vector, such as

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antibiotic markers, ease of recovery of the desired protein, expression characteristics of the DNA sequences and the expression control sequences operatively linked to them, biosafety, costs and the  
5 folding, form or any other necessary post-expression modifications of the desired polypeptide.

While recombinant DNA techniques are the preferred method of producing the polypeptides of this invention having a sequence of more than 20 amino acids, shorter polypeptides encompassed by this invention having less than about 20 amino acids are preferably produced by conventional chemical synthesis techniques. Synthetically produced polypeptides of this invention can advantageously be obtained in  
10 extremely high yields and be easily purified.

In a preferred embodiment of this invention, the polypeptides are synthesized by solution phase or solid phase polypeptide synthesis and, optionally, digested with carboxypeptidase (to remove C-terminal  
20 amino acids) or degraded by manual Edman degradation (to remove N-terminal amino acids). Proper folding of the polypeptides may be achieved under oxidative conditions which favor disulfide bridge formation, as described by S.B.H. Kent, "Chemical synthesis of  
25 polypeptides and proteins", Ann. Rev. Biochem., 57, pp. 957-89 (1988). Polypeptides produced in this way may then be purified by separation techniques widely known in the art, preferably utilizing reverse phase HPLC. The use of solution phase synthesis  
30 advantageously allows for the direct addition of certain derivatized amino acids to the growing polypeptide chain, such as the O-sulfate ester of tyrosine. This obviates the need for a subsequent derivatization step to modify any residue of the  
35 polypeptides of this invention.

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The biological activity of the polypeptides of this invention, i.e., their ability to block LFA-3/CD2 interaction, may be assayed using a simple cell binding assay that permits visual (under 5 magnification) evaluation of the binding of LFA-3 polypeptide-expressing cells to CD2-expressing cells. Jurkat cells are preferred as the CD2<sup>+</sup> substrate (see Examples, *infra*.). The binding characteristics of soluble polypeptides according to the invention may be 10 assayed in several known ways, such as by radiolabeling the polypeptide with, e.g., <sup>35</sup>S, and then contacting the labeled polypeptide with CD2<sup>+</sup> cells. Enzymatic labels or rosetting assays such as described by Seed et al. (Proc. Natl. Acad. Sci. USA, **84**, pp. 3365-69 (1987)) 15 may also be used.

In another embodiment of this invention, fusion proteins and DNA sequences coding for them are provided. These fusion proteins have an amino-terminal region characterized by the amino acid sequence of a 20 CD2-binding polypeptide of this invention and a carboxy-terminal region comprising a domain of a protein or polypeptide other than LFA-3. Such domains include, for example, the Fc region of an immunoglobulin.

25 In the preferred fusion proteins of this invention, the CD2-binding polypeptides of this invention are fused to at least a portion of the Fc region of an immunoglobulin. In these fusion proteins, the CD2-binding polypeptides form the amino-terminal 30 portion, and the Fc region forms the carboxy terminal portion.

In these fusion proteins, the Fc region is preferably limited to the hinge region and the C<sub>H</sub>2 and C<sub>H</sub>3 domains. More preferably, the Fc region in the 35 fusion proteins of this invention is limited to a

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portion of the hinge region, the portion being capable of forming intermolecular disulfide bridges, and the C<sub>H</sub>2 and C<sub>H</sub>3 domains. See, e.g., Figure 12.

An example of a useful LFA-3-Ig fusion

5 protein of this invention is LFA3TIP (also referred to as LFA-3(92)IgG), which is secreted into the cell culture medium by COS7 cells containing the expression plasmid pSAB152 (see *infra*). LFA3TIP consists of the amino terminal 92 amino acids of the mature form of  
10 LFA-3 fused to a portion of the hinge region and the C<sub>H</sub>2 and C<sub>H</sub>3 constant domains of human IgG1 (see Figure 11). The fusion protein, LFA3TIP, contains a functional CD2-binding domain of LFA-3 and a sufficient portion of the Fc region of IgG to be recognized by the  
15 Fc binding protein, Protein A. LFA3TIP is able to bind to CD2 and inhibit T-cell activation.

It will be apparent that the polypeptides and fusion proteins of this invention may be used in methods to selectively isolate CD2 or CD2-expressing  
20 cells, based on the formation of a complex between the CD2-binding domain of those polypeptides and CD2.

In another embodiment of this invention, the polypeptides and fusion proteins of this invention, such as the LFA-3-Ig fusion proteins, may be used to  
25 label cells expressing CD2 or a polypeptide containing the LFA-3 binding domain of CD2. For example, the polypeptides and fusion proteins of this invention may be conjugated to a "reporter molecule" which allows detection of the polypeptides or fusion proteins bound  
30 to CD2<sup>+</sup> cells or polypeptides containing the CD2-binding domain of LFA-3. Owing to the presence of a portion of the immunoglobulin Fc region in the illustrative fusion proteins of this invention, such fusion proteins can be conjugated to the same "reporter  
35 molecules" which are commonly conjugated or bound to

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immunoglobulins in order to detect immunoglobulins bound to antigens, e.g.,  $^{125}\text{I}$ , enzyme-conjugated secondary antibodies directed to the Fc region, or biotin-streptavidin based enzyme conjugated molecules.

5 Accordingly an LFA-3-Ig fusion protein of this invention may be conjugated or bound to such reporter molecules via its Fc carboxy-terminal portion. Furthermore, such reporter molecules may be conjugated or bound to the polypeptides or fusion proteins of this  
10 invention before or after the polypeptides or fusion proteins have bound to CD2 or to the LFA-3 binding domain of CD2.

It is a further embodiment of this invention that the polypeptides and fusion proteins of this  
15 invention can also be used in diagnostic applications to detect the presence or indicate the absence of CD2 or cells or molecules containing the LFA-3-binding domain of CD2.

The polypeptides and fusion proteins of this  
20 invention can also be used in therapeutic compositions to inhibit formation of the CD2/LFA-3 complex, when such formation contributes to a pathological state. Alternatively, they may be used therapeutically to mimic the role of LFA-3 in initiating one or more of  
25 the T-lymphocyte functional responses dependent on the formation of the CD2/LFA-3 complex (see, e.g., Dustin et al., J. Exp. Med., 165, pp. 677-92 (1987); Springer et al., Ann. Rev. Immunol., 5, pp. 223-52 (1987)). For example, the polypeptides and fusion proteins of this  
30 invention may be used in the treatment of acute and chronic inflammation, autoimmune diseases, and for immunomodulation, including treatment of such diseases as systemic lupus erythematosus or lupus vulgaris, rheumatoid arthritis and thyroiditis. Furthermore, the  
35 polypeptides and fusion proteins of this invention may

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be used to inhibit T-cell activation or to inhibit proliferation of peripheral blood lymphocytes.

In these respects, it is recognized that molecules involved in cell-cell adhesion are generally 5 more effective in eliciting a particular response from a cell when the molecules are present in a multimeric form as opposed to a monomeric form of the same protein. Multimeric forms of cell surface adhesion proteins appear to more closely mimic the typical 10 situation in vivo where, e.g., an effector cell will exhibit hundreds or thousands of copies of a particular adhesion protein on its surface which then bind to the many copies of its ligand on a target cell. When many surface molecules become involved in binding, they may 15 act synergistically, so that the affinity of one cell for another is greater than the mere sum of the binding affinities of the individual molecules. Accordingly, an important aspect of this invention concerns the preparation and use of multimeric forms of the CD2- 20 binding polypeptides disclosed herein.

A variety of methods are known in the art to form multimeric forms of protein monomers. Such methods include using crosslinking agents, e.g., glutaraldehyde (e.g., Reichlin, Methods Enzymol., 70, 25 pp. 159-65 (1980)). If thiol residues are present on a polypeptide or polypeptides, such groups may be oxidized to form intermolecular disulfide bonds to achieve multimeric forms of the polypeptide or polypeptides. Thiol residues or thiol-reactive groups 30 may be introduced into a polypeptide using iminothiolane or heterobifunctional cross-linkers, such as N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP), which contains an amine-reactive group and a thiol-reactive group. Coupling of the proteins may then be 35 accomplished through disulfide bonds formed either

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directly or through homobifunctional cross-linking agents (see, e.g., Srinivasachar et al., Biochem., 28, pp. 2501-09 (1989); Ramakrishnan et al., Cancer Res., 44, pp. 201-08 (1984); Lambert et al., J. Biol. Chem.,

5 260, pp. 12035-41 (1985)). The effectiveness of disulfide bond formation between molecules would of course be limited to the number of thiols available on the polypeptide (naturally occurring or introduced by derivatization as above) and whether such disulfide  
10 bond formation adversely affected the affinity of the resulting multimeric form.

If polypeptides or proteins possess carbohydrate groups, such as in glycoproteins, the sugar moieties of such groups may be used in reactions  
15 to link one molecule with another (e.g., Liao et al., J. Biol. Chem., 248, pp. 8247-53 (1973); Moroney et al., Biochem., 26, pp. 8390-98 (1987)).

Other multimeric forms of the CD2-binding polypeptides of this invention may be produced by  
20 attaching a phosphatidylinositol ("PI") linkage sequence, e.g., as described in PCT patent application PCT/US90/01859, or sequences of C4 binding protein, e.g., as described in PCT patent application PCT/US91/00567. The hydrophobic PI anchor may be used  
25 to form micelles exhibiting a plurality of active CD2-binding domains or multimeric forms of the PI-linked LFA-3, in which a plurality of monomers are associated with one another by hydrophobic interactions between the PI linkage sequences of each monomer (in  
30 the absence of added lipids or membrane). In this latter case, multimeric PI-linked LFA-3 molecules are usually octamers; though other polymeric forms have been observed (e.g., 6-12 associated monomers). Alternatively, attaching a segment of DNA encoding the  
35 PI linkage sequence downstream of a DNA insert encoding

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a polypeptide of the invention, and transfecting suitable mammalian host cells with this construct, will provide a culture of cells exhibiting numerous copies of the CD2-binding polypeptide on their surfaces

5 (attached by a PI anchor).

Alternatively, multiple copies of monomers of polypeptides and fusion proteins of this invention may be bound to another molecule or substrate or particle.

As in the case of the binding of LF08 to Affigel-10

10 beads (see *infra*), the formation and use of molecules, compounds or particles comprising multiple CD2-binding domains are within the scope of this invention.

In addition, this invention also includes multimeric forms of LFA-3-Ig fusion proteins. Such

15 multimers may be generated by using those Fc regions, or portions thereof, of Ig molecules which are usually multivalent, e.g., IgM pentamers and IgA dimers. It is of course understood that a J chain polypeptide may be necessary to form and stabilize IgM pentamers and IgA

20 dimers. Alternatively, multimers of LFA-3-Ig fusion proteins may be formed by using a protein with an affinity to the Fc region of Ig molecules, such as Protein A. For example, a plurality of LFA-3-Ig fusion proteins, such as LFA3TIP, may be bound to Protein A-  
25 agarose beads to form agarose beads whose surfaces are covered with multiple functional CD2-binding domains of the attached LFA-3-Ig fusion proteins.

In another embodiment, this invention provides multimeric proteins capable of binding to CD2, 30 which comprise (a) two or more of the CD2-binding polypeptides described herein, (b) two or more of the CD2-binding fusion proteins described herein, or (c) one or more of the CD2-binding polypeptides and one or more of the CD2-binding fusion proteins.

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The polypeptides of this invention may also be used to design low molecular weight (i.e., usually monomeric molecular weights of less than 1000 daltons) nonpeptidyl or only partial peptidyl CD2-binding molecules useful to inhibit cell-cell adhesion via formation of the CD2/LFA-3 complex. Based on the polypeptides provided herein, such small molecules may be designed which possess the ability to bind CD2 and may consist of moieties other than amino acids and may be produced entirely through synthetic methods. The use of such small molecules as therapeutic or diagnostic agents also falls within the scope of this invention.

Another embodiment of this invention involves the use of the CD2-binding polypeptides and fusion proteins to obtain antibodies recognizing the CD2-binding domain of LFA-3. Both monoclonal antibodies (MAbs) and polyclonal antibodies highly specific to the CD2-binding domain of LFA-3 may be obtained utilizing the polypeptides and fusion proteins of this invention.

Methods for obtaining monoclonal antibodies and polyclonal antiserum to a particular antigen are well known in the art. For producing MAbs, an immortal cell line (typically a myeloma cell line) is fused to lymphocytes (typically splenocytes) from a mammal (e.g., a rabbit) immunized with a particular antigen, i.e., in this case the CD2-binding domain of LFA-3. Such fusions usually generate hybridomas, i.e., immortal clones of hybrid cells which produce antibody molecules specific for a single epitope of the immunizing antigen (see, generally, Kohler et al., Nature, 256, pp. 495-97 (1975)). Effective immunization may require that the polypeptide comprising the CD2-binding domain be polymerized or derivatized and mixed with an adjuvant.

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It is also necessary to be able to screen the potentially numerous clones of hybridomas generated from the fusions in order to identify those clones which produce antibodies to the CD2-binding domain of LFA-3. For example, such screens may involve assaying the supernatants of cultures of hybridomas for the ability to inhibit CD2-expressing cells from binding to LFA-3 expressing cells. Assays which have been used to screen hybridomas for the production of MAbs to LFA-3 are applicable as primary screens for hybridomas producing MAbs specific for the CD2-binding domain of LFA-3 (see, e.g., Sanchez-Madrid et al., Proc. Natl. Acad. Sci. USA, 79, pp. 7489-93 (1982)).

The DNA encoding the CD2-binding polypeptides of this invention and of deletion mutant forms of the LFA-3 gene, such as those described herein, may also be used as plus/minus probes to detect and isolate other DNA sequences encoding the CD2-binding domain of LFA-3.

The deletion mutant forms of the LFA-3 protein, which lack at least amino acids +51 to +60 of mature LFA-3; at least amino acids +41 to +50 of mature LFA-3 or at least amino acids +31 to +40 of mature LFA-3; (and preferably lack all 3 regions) may be used to clear polyclonal antiserum of antibodies which recognize epitopes of LFA-3 other than the epitopes within the CD2-binding domain of LFA-3. Such mutants may be, for example, selected from the group consisting of M57, M55, M56, PIM3 and M100, and combinations thereof. For example, a polyclonal anti-LFA-3 antiserum may be preincubated with a mutant form of the LFA-3 protein encoded by the mutant LFA-3 gene containing the M57 or M100 deletion mutations (Figure 1 and Figure 6). The polyclonal antibodies recognize and bind LFA-3 epitopes on the mutant LFA-3 protein. However, since the CD2-binding domain is not present,

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antibodies to this epitope in the polyclonal antiserum will not bind. Precipitation of the complexes that do form will result in an enrichment for any antibodies remaining in the antiserum that are specific for

5 epitopes of the CD2-binding domain.

The CD2-binding domain polypeptides and fusion proteins of this invention may be formulated as pharmaceutical compositions using conventional methods to prepare pharmaceutically useful compositions and 10 combinations. Such compositions preferably include at least one pharmaceutically acceptable carrier. See, e.g., Remington's Pharmaceutical Sciences, (E.W. Martin). Pharmaceutical compositions of the present invention typically contain, in addition to the active 15 polypeptide, a pharmaceutically acceptable buffer, preferably phosphate buffered saline, together with a pharmaceutically acceptable compound for adjusting isotonic pressure, such as sodium chloride, mannitol or sorbitol. The pharmaceutically acceptable compositions 20 and methods of this invention are characterized by pharmaceutically effective amounts of a polypeptide according to the invention.

The pharmaceutical compositions and combinations of this invention which comprise LFA-3-Ig 25 fusion proteins may be used, for example, to inhibit the activation of T-lymphocytes or to inhibit the proliferation of peripheral blood lymphocytes (see infra).

The term "combination" as used herein, 30 includes a single dosage form containing at least one polypeptide of this invention and at least one other pharmaceutically active agent, a multiple dosage form wherein the polypeptide and the other active agent are administered separately, but concurrently, or a 35 multiple dosage form wherein the two components are

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administered separately but sequentially.

Alternatively, the polypeptides of this invention and the other active agent may be in the form of a single conjugated molecule. Conjugation of the two components

5 may be achieved by standard cross-linking techniques well known in the art. A single molecule may also take the form of a recombinant fusion protein.

The pharmaceutical compositions and combinations of this invention may be administered to a  
10 patient in any pharmaceutically acceptable dosage form, including, but not limited to, those which may be administered to a patient intravenously as a bolus or by continued infusion, intramuscularly, subcutaneously, intracutaneously, intra-articularly, orally or  
15 parenterally.

Methods for determining pharmaceutically effective dosages are known to those skilled in the art. The dosage and dose rate will depend on a variety of factors such as the specific composition, the object  
20 of the treatment, i.e., therapy or prophylaxis, method of administration, and the judgment of the treating physician.

This invention also relates to the bioanalytic use of CD2-binding polypeptides and fusion  
25 proteins, or compositions containing them, for determining the concentration of CD2 proteins or the detection of CD2-expressing cells in a biological sample. These polypeptides and compositions may be used in a manner similar to that of reagents employed  
30 in conventional assays. In addition, the polypeptides of this invention may be utilized in diagnostic kits designed to detect the presence and measure the concentration of CD2 or CD2-expressing cells.

In order that this invention may be better  
35 understood, the following examples are set forth.

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These examples are for purposes of illustration only, and are not to be construed as limiting the scope of this invention.

EXAMPLE 1

5 Preparation of Deletion Mutations In the LFA-3 Gene

In order to map the CD2-binding domain on the LFA-3 protein, a series of deletion mutations of the LFA-3 gene were systematically generated using site-specific mutagenesis by oligonucleotide

10 heteroduplexing. cDNA encoding transmembrane has been isolated and sequenced (see, Wallner et al., United States Patent 4,956,281), and a bacteriophage bearing cDNA coding for transmembrane LFA-3 was deposited with American Type Culture Collection in Rockville, Maryland

15 (accession number 75107). A plasmid, pHT16-6, containing cDNA encoding transmembrane LFA-3 was obtained from Biogen, Inc. (Cambridge, Massachusetts) and used in preparing all the deletion mutations. The construction of plasmid pHT16-6 is described in PCT patent publication WO 88/09820, which is herein incorporated by reference. Plasmid pHT16-6 contains a cDNA insert encoding LFA-3 and contains a unique EcoRI site near the 5' end of the LFA-3 coding sequence, a unique BamHI site adjacent the 5' end of the LFA-3

20 25 cDNA, a unique HindIII site adjacent the 3' end of the LFA-3 cDNA, two NotI sites 1112 bp apart and bracketing the LFA-3 cDNA, and a unique ScaI site. The cDNA insert of pHT16-6 containing the entire coding region for transmembrane LFA-3 is shown in Figure 1.

30 In this procedure, seventeen sequences of the LFA-3 coding sequence, each 30 bp long, were selected for deletion. For each proposed deletion, a 30-base antisense oligonucleotide (30-mer) was synthesized which consisted of the complementary 15 bases

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immediately upstream of (5' to) the proposed deletion and the complementary 15 bases immediately downstream of (3' to) the proposed deletion. Hybridization of a particular synthetic oligonucleotide to a strand of the

5 LFA-3 DNA resulted in a heteroduplex in which a specific sequence of 30 bp would loop out. By this mutagenesis procedure, some of the replication products of the heteroduplex contained deletion mutations lacking the 30 bp region looped out by heteroduplex

10 formation.

The synthetic oligonucleotides used to generate the seventeen deletion mutants depicted in Figure 1 are set forth in the following table:

	<u>Sequence</u>	Complementary to <u>LFA-3 Nucleotides</u>	Segment Deleted
15	SEQ ID NO:13	109-123+154-168	M53
	SEQ ID NO:14	139-153+184-198	M54
	SEQ ID NO:15	169-183+214-228	M55
	SEQ ID NO:16	199-213+244-258	M56
20	SEQ ID NO:17	229-243+274-288	M57
	SEQ ID NO:18	259-273+304-318	M58
	SEQ ID NO:19	289-303+334-348	M59
	SEQ ID NO:20	319-333+364-378	M60
	SEQ ID NO:21	349-363+394-408	M61
25	SEQ ID NO:22	379-393+424-438	M62
	SEQ ID NO:23	409-423+454-468	M63
	SEQ ID NO:24	439-453+484-498	M64
	SEQ ID NO:25	469-483+514-528	M65
	SEQ ID NO:26	499-513+544-558	M66
30	SEQ ID NO:27	529-543+574-588	M90

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SEQ ID NO:28	559-573+604-618	M91
SEQ ID NO:29	589-603+634-648	M92.

For each heteroduplex, one sample of plasmid pHT16-6 (100 µg) was digested with HindIII (210 units, 5 New England Biolabs) and either EcoRI (300 units, New England Biolabs) or BamHI (300 units, New England Biolabs). Another sample of pHT16-6 (100 µg) was linearized by digestion with ScaI (300 units, New England Biolabs). Digestion products were 10 electrophoresed on 1 percent (w/v) agarose gels in TAE buffer (40 mM Tris/acetate, 1 mM EDTA, pH 8.0), and the desired fragment from each digestion was then electroeluted from the gel (Maniatis et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, 15 1982)). In the case of the ScaI digestion, the linearized pHT16-6 was electroeluted from the gel; in the case of the HindIII/EcoRI or HindIII/BamHI digestions of pHT16-6, the larger of two restriction fragments produced in each case was electroeluted. 20 A sample of the ScaI linearized pHT16-6 DNA (100 ng) was mixed with a sample of the isolated HindIII/EcoRI or HindIII/BamHI restriction fragment (100 ng) and with 8 picomoles of one of the seventeen antisense oligonucleotides previously phosphorylated 25 with ATP (Maniatis et al., supra). Referring to Figure 1, since the EcoRI restriction site is located in the M56 region, the HindIII/EcoRI large restriction fragment could not be used to generate deletions of regions M53, M54, M55 and M56. Thus, the HindIII/BamHI 30 large restriction fragment was used in the procedure to generate these four deletions.

In order to form heteroduplexes, a synthetic oligonucleotide, ScaI-linearized pHT16-6, and either HindIII/EcoRI or HindIII/BamHI large fragment of

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pHT16-6 were mixed in 100 mM NaCl, 80 mM MgCl<sub>2</sub>, 6.5 mM Tris/Cl, pH 7.6 and boiled for 3 minutes to denature. The denatured DNA was allowed to anneal at 37°C for 1 hour, then at 4°C for 1 hour, and finally at 0°C for 10

5 minutes.

After annealing, the gaps in the heteroduplexes were filled in and the fragments ligated in a single reaction mixture by adding to the annealed DNA the following constituents to give the indicated

10 final concentrations or amounts: ATP (20 mM); dATP, dTTP, dGTP, dCTP (each at 10 mM); Klenow (large fragment of DNA Polymerase I, 2.5 units, New England Biolabs); T4 DNA ligase (200 units, New England Biolabs). The mixtures were then incubated overnight

15 at 15°C.

After incubation, half of each fill-in/ligation mix was used to transform 100 µl of E.coli JA221 (Maniatis et al., supra). Colonies of transformed cells were selected on LB plates

20 supplemented with ampicillin (50 µg/ml) (Maniatis et al., supra).

Colonies of transformants were screened for the presence of a particular deletion by colony hybridization using the particular oligonucleotide used

25 in the heteroduplex to generate the deletion as a probe.

For use as probes, a sample of each oligonucleotide (80 pmol) was labeled using <sup>32</sup>P-ATP by standard procedures. The labeled oligonucleotides were

30 precipitated with 2 M ammonium acetate (Maniatis et al., supra). Labeled probe was added to the

hybridization buffer at a final activity of

approximately  $5 \times 10^5$  CPM/ml of hybridization mixture.

Hybridizations were carried out on nitrocellulose

35 filters at 60°C for about 8 hours. After washing the

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filters in 0.5 x SSC buffer at 60°C, autoradiography was performed and positive colonies scored (Maniatis et al., *supra*). The generation of the desired deletion mutations was confirmed by DNA sequence analysis (Maxam 5 and Gilbert, in Methods in Enzymology (Grossman and Moldave, eds.), 65, pp. 499-560 (1980)) of plasmid DNA in colonies scoring positive according to hybridization results.

These procedures produced plasmids, each 10 containing one of the 30 bp deleted sequences listed above and depicted in Figure 1. The plasmid containing the M57 deletion in the LFA-3 coding sequence was designated plasmid pLFA3M57-4A.

EXAMPLE 2

15 Construction of Recombinant Expression Plasmids

Plasmids prepared according to Example 1 with successfully deleted 30 bp segments from the LFA-3 coding sequence were used for construction of expression vectors. Aliquots of 100 µg of plasmids 20 having deleted segments (see Figure 1) M54, M55, M56, M57, M58, M63 and M65, respectively, were digested with NotI endonuclease (100 units, New England Biolabs, Beverly, Massachusetts). For M57, M63 and M65, the 5' overhangs were filled in using a standard Klenow 25 (New England Biolabs) reaction mix (Maniatis et al., *supra*) to generate blunt ended restriction fragments. The fragments were purified from 0.7% TAE agarose gels by electroelution.

The gel-purified, blunt-ended, NotI fragments 30 were then individually cloned into the SmaI site of the eukaryotic expression vector pBG368PY (Dailey et al., *J. Virol.*, 54, pp. 739-49 (1985)) using T4 ligase at 15°C overnight. NotI fragments of M54, M55, M56, and M58 were gel purified as described above and ligated to

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NotI-digest expression vector, PMDR902. The resulting ligations were used to transform E.coli JA221 and positive colonies of transformants containing the recombinant plasmids carrying the desired inserts were

5 identified by colony hybridization, as described above. The orientation of the M57, M63 and M65 inserts in the vector was confirmed by EcoRI digestion, generating 6600, 4000 and 200 bp fragments for correctly oriented inserts, which were separated on 0.7% agarose gel. For 10 plasmids containing M54, M55, M56 and M58 inserts, the orientation was determined by PvuII digestion analysis, where the correct orientation generated fragments of 6599 bp, 1185 bp and 506 bp. All fragments were verified by DNA sequencing (Maxam and Gilbert, supra).

15 The recombinant expression plasmids carrying DNA encoding the M54, M55, M56, and M58 LFA-3 deletion mutations were linearized with AatII and electroporated into Chinese Hamster Ovary (CHO) cells according to the published protocol of J. Barsoum (DNA Cell Biol., 9, 20 pp. 293-300 (1990)). Plasmids carrying DNA encoding the M57, M63 and M65 deletion mutations were linearized using NruI and electroporated into CHO cells as above, except that 19  $\mu$ g of the NruI-digested DNA was ethanol precipitated overnight at -20°C with 1  $\mu$ g of EcoRI- 25 digested SV2 DNA (DHFR $^+$ ) and 380  $\mu$ g of sonically disrupted salmon sperm DNA. CHO cells ( $1 \times 10^7$ ) were electroporated with the coprecipitated DNA at 280 volts using a BioRad Gene Pulser.

After electroporation, cells were seeded into 30 two 100 mm plates in alpha $^+$  MEM, 10% fetal calf serum (FCS), 4 mM L-glutamine, penicillin/streptomycin, then incubated for 48 hours at 37°C. The cells in the plates were then divided into five 100 mm plates containing alpha $^-$  MEM, 10% FCS, 4 mM L-glutamine, 35 penicillin/streptomycin, and incubated for five days at

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37°C. The cells were then fed with alpha<sup>-</sup> Complete Medium supplemented with 200 nM methotrexate for M57, M63 and M65, and 50 nM methotrexate for M54, M55, M56 and M58. Each plate was grown to 90% confluence then 5 assayed by FACS (Fluorescence-Activated Cell Sorter) to determine whether any of the CHO cultures transfected with the LFA-3 deletion mutations expressed the mutant forms of LFA-3.

EXAMPLE 3

10 FACS Analysis of Transfected Cells

Transfected CHO cells, prepared in Example 2 and grown in the presence of methotrexate to 90% confluence, were rinsed twice with HANKS BSS medium (Ca<sup>++</sup> and Mg<sup>++</sup> free, pH 7.0) and removed with HANKS 15 (Ca<sup>++</sup> and Mg<sup>++</sup> free, 5 mM EDTA, pH 7.0) medium. Approximately 2 x 10<sup>5</sup> cells were resuspended in 100 µl of a solution of cold (0°C) wash buffer (PBS, 0.1% NaN<sub>3</sub>, 0.5% bovine serum albumin (BSA), pH 7.2), and a primary antibody was added. The primary antibody was 20 either anti-LFA-3 MAb TS2/9 ascites fluid (see, e.g., Sanchez-Madrid et al., Proc. Natl. Acad. Sci. USA, 79, pp. 7489-93 (1982), obtained from Dana Farber Cancer Inst., Boston, Massachusetts); the anti-LFA-3 MAb 7A6 (obtained from Biogen, Inc., Cambridge, MA; 1.6 µg/ml); 25 or anti-LFA-3 polyclonal rabbit antiserum 202 (obtained from Biogen, Inc.). All the primary antibodies are known to block CD2 adhesion to LFA-3. The amount of primary antibody used was typically between 1 and 2 µl. Cells were incubated at 0°C for 45 minutes and washed 30 twice with wash buffer.

A fluorescein-labeled secondary antibody was then added and the mixture incubated for 30 minutes at 0°C. For analysis with the monoclonal antibodies, the

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secondary antibody was fluorescein (DTAF)-conjugated affinity purified goat anti-mouse F(ab)<sub>2</sub> IgG (H+L) (Jackson Immunoresearch Laboratories, Inc., West Grove, CA); for use with the polyclonal rabbit antiserum 202,  
5 the secondary antibody was fluorescein isothiocyanate (FITC) goat anti-rabbit IgG (H+L) (Fisher Biotech, Pittsburgh, Pennsylvania).

After incubation with the secondary antibody, cells were overlayed with 300 µl FCS, washed twice in  
10 wash buffer, resuspended in 300 µl 1 x PBS and transferred to Falcon 2052 tubes to be read on the cell sorter.

Referring to Figure 2, the FACS analyses indicated that cells transfected with expression  
15 vectors containing deletion mutations M57 (Figures 2A and 2B), M65 (Figures 2C and 2D), M63 (Figures 2E and 2F), M54 (Figures 2G and 2H), M55 (Figures 2I and 2J), M56 (Figures 2K and 2L), and M58 (Figures 2M and 2N) expressed a surface protein that was recognized by the  
20 anti-LFA-3 polyclonal antiserum 202 (Figures 2A, 2C, 2E, 2G, 2I, 2K, 2M). However, the anti-LFA-3 MAbs TS2/9 and 7A6 bound only to transfectants carrying the M65 and M63 deletion mutants and not to transfectants lacking the M57, M54, M55, M56, or M58 regions (compare  
25 Figures 2B, 2H, 2J, 2L, 2N with 2D, 2F). These results indicated that the M54, M55, M56, M57 and M58 segments, which were deleted from the LFA-3 protein in, respectively, the deletion mutations M54, M55, M56, M57 and M58 (see Figure 1), were important in CD2  
30 recognition of the LFA-3 molecule.

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EXAMPLE 4

Jurkat Cell Binding Assay

CHO cells transfected with the M57 deletion mutation ("M57/CHO" cells), CHO cells transfected with plasmid p24 (see, Wallner et al., PCT patent application WO 90/02181) expressing PI-linked LFA-3 ("P24/CHO" cells, positive control), and normal CHO cells (negative control) were tested for the ability to bind Jurkat cells expressing CD2.

10 M57/CHO cells, P24/CHO cells and CHO cells ( $1 \times 10^5$ ) were added to separate wells of a 6-well plate (Corning) and washed twice with RPMI Complete Medium. Jurkat cells ( $CD2^+$ ), obtained as a gift from Dr. Timothy Springer (Dana Farber Cancer Inst., Boston, Massachusetts) were washed three times with a solution of RPMI, 10% FCS, 4 mM L-glutamine, and then  $5 \times 10^6$  cells were added to each well and incubated for 4 hours at 0°C. Cells were then washed gently three times with RPMI and examined under the microscope at 40X and 100X magnification for cell-cell binding.

15 Referring to Figure 3, whereas positive control cells expressing PI-linked LFA-3 bound Jurkat cells expressing CD2 (Figure 3C), M57/CHO cells failed to bind Jurkat cells (Figure 3B), as did nontransfected (LFA-3<sup>-</sup>) CHO cells (Figure 3A).

EXAMPLE 5

Northern Blot Analysis of mRNA from M57/CHO Cells

The mRNA of M57/CHO cells was analyzed by Northern blot as a further confirmation that the failure of the monoclonal antibodies, i.e., MAbs TS2/9 and 7A6, to bind to the M57/CHO cells was due to the surface LFA-3 mutant lacking the M57 CD2-binding region

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rather than to inefficient expression of the mutant LFA-3 gene.

RNA from about  $1 \times 10^7$  M57/CHO cells on a 100 mm plate was isolated as follows: Growth media were 5 removed and 2 ml extraction buffer (50 mM Tris-Cl (pH 7.5), 1% SDS, 5 mM EDTA, proteinase K added to 100  $\mu\text{g}/\text{ml}$  immediately prior to use) was added. The plate was incubated 20 minutes at 37°C with gentle shaking. A viscous cell lysate developed and was collected in a 10 50 ml test tube. An equal volume of phenol:chloroform:ether (50:50:1) was added and the mixture vortexed briefly. The mixture was homogenized on a Polytron mixer (Kinematica, Switzerland) for 15 seconds at highest speed to shear the DNA. The mixture 15 was poured into a 15 ml Corex tube and centrifuged at 10,000 rpm, 10 minutes at 4°C.

After centrifuging, the aqueous phase was collected and NaCl was added to 0.25 M, followed by 1 volume of isopropanol. This mixture was placed on dry 20 ice for 10 minutes, defrosted and centrifuged 15 minutes at 10,000 rpm and 4°C.

The pellet was resuspended in 2.9 ml distilled water and vortexed. 0.9 ml of 12 M LiCl was added (to 2.8 M) and the suspension allowed to stand at 25 least four hours at 4°C. 5S RNA and DNA remained in solution; other RNA fractions precipitated. This solution was centrifuged for at least 15 minutes at 10,000 rpm at 4°C.

The pellet was resuspended in 360  $\mu\text{l}$  30 distilled water and transferred to an Eppendorf tube. 40  $\mu\text{l}$  3 M NaAc (pH 5.2) and 1 ml ethanol were added, then the mixture was cooled at -70°C for 5 minutes, cooled at -20°C for 15 minutes, centrifuged, rinsed with ethanol, and resuspended in 400  $\mu\text{l}$  0.3 M NaAc. 35 Ethanol (1 ml) was added and the precipitation was

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repeated. The RNA recovered after centrifuging was resuspended 100  $\mu$ l distilled water.

10  $\mu$ g of the RNA were electrophoresed on a 1% agarose-formaldehyde gel, transferred to a GeneScreen 5 nitrocellulose membrane (New England Nuclear) and hybridized to a  $^{32}$ P-labeled probe. As a control, RNA extracted from the cell line M16.3/CHO (expressing recombinant soluble LFA-3, obtained from Biogen, Inc., Cambridge, Massachusetts) was analyzed on the same 10 Northern blot. The Northern blot analysis (Figure 4) indicated efficient synthesis of the M57 RNA in the M57/CHO cells.

EXAMPLE 6

Immunoprecipitation of LFA-3 Deletion Mutant

15 To verify the loss of specific antibody binding domains in the mutant LFA-3 expressed on the surface of M57/CHO cells, M57/CHO transfectants, untransfected CHO cells, and P24/CHO transfectants (which express PI-linked LFA-3) were surface labeled 20 with  $^{125}$ I, and each of the three primary antibody preparations (i.e., polyclonal antiserum 202, MAb TS2/9 and MAb 7A6) were used to immunoprecipitate LFA-3 after detergent disruption of the surface membrane.

About  $10^7$  (each) of M57/CHO cells, normal CHO 25 cells and P24/CHO cells suspended in PBS<sup>\*</sup> (i.e., PBS without Ca<sup>++</sup> and Mg<sup>++</sup>) supplemented with 5 mM EDTA were washed three times with PBS<sup>\*</sup> and resuspended in 0.5 ml PBS. Washed cells were added to glass tubes (12 x 75 mm<sup>2</sup>) previously coated with a solution of 50  $\mu$ g 30 1,3,4,6-tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenylglycoluril (Iodo-Gen, Pierce Bio-Research, Rockford, Illinois) in 100  $\mu$ l CHCl<sub>3</sub> and dried in nitrogen. Just before use, the tubes were rinsed with PBS, and then  $^{125}$ Iodine (1 mCi)

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was added and the tubes incubated on ice for 30 minutes, with swirling every 10 minutes.

After incubation, the iodinated cells were added to 10 ml alpha<sup>-</sup> MEM, 10% FCS, 4 mM L-glutamine.

- 5 The cells were spun down, washed twice with 5 ml of the same medium, and then resuspended in 1 ml PBS and centrifuged again. The washed cells were then lysed by resuspension in 1 ml DOC buffer (20 mM Tris/Cl, pH 7.3; 50 mM NaCl, 0.5% deoxycholate (DOC); 0.5% NP40)
- 10 containing 20 µl of protease inhibitor PMSF (Sigma Chemical Co., St. Louis, Missouri, 17 mg/ml in ethanol), followed by incubation for 30 minutes on ice. The lysate was then microcentrifuged for 10 minutes at 4°C, and the supernatants containing the <sup>125</sup>I-labeled
- 15 surface molecules removed.

Lysates from the foregoing iodinations were precleared by contacting with 50 µl of protein A-Sepharose and incubating on a rocker for 1 hour at room temperature. 100 µl of lysate was removed for each sample and mixed with 300 µl of DOC buffer. This mixture was centrifuged and the resulting cleared supernatant removed to a new tube.

- 20 Primary antibody was added to samples of each of the cleared supernatants: Polyclonal anti-LFA-3 antiserum 202 was used at a final concentration 40 µg/ml; MAbs TS2/9, 7A6 and a control monoclonal antibody, MOPC-21 (IgG1, not specific to LFA-3) were used at 5 µg/ml. 100 µl DOC and 2 µl protease inhibitor PMSF were added, and the mixtures incubated
- 25 on a rocker for 2 hours at room temperature 25°C or overnight at 4°C.

After incubation, 30 µl of protein A-Sepharose (for polyclonal antiserum 202 samples) or 15 µl of anti-mouse IgG-agarose (Sigma Chemical Co.)

- 30 (for MAb samples) was added to the samples, followed by

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incubation on a rocker for 2 hours at room temperature. The mixtures were then centrifuged for 2 minutes, and the supernatants carefully removed and discarded. 1 ml of DOC buffer was then added, and the pellets  
5 resuspended by vortexing. The resuspended material was then washed 3 times with DOC buffer.

To prepare the samples for analysis by polyacrylamide gel electrophoresis, 30  $\mu$ l of SDS sample buffer was added, the sample heated at 65°C for 15  
10 minutes. The sample was then centrifuged for 2 minutes, and the supernatant removed and saved for electrophoresis. 15  $\mu$ l of the supernatant was run on a denaturing 10-20% gradient polyacrylamide gel (Daiichi system, Enprotech), and autoradiography performed to  
15 identify immunoprecipitated surface proteins.

Figure 5 is an autoradiograph of an SDS polyacrylamide gel showing antibody-precipitated LFA-3 surface proteins from M57/CHO, normal CHO, and P24/CHO cells. This gel revealed that TS2/9, 7A6 and the  
20 polyclonal antiserum 202 precipitated LFA-3 from the CHO cells expressing the PI-linked LFA-3 (lanes 9, 10, 11). In contrast, only polyclonal antiserum 202 precipitated the LFA-3 surface protein from M57/CHO cells expressing the deletion mutant M57 LFA-3 gene  
25 (lane 4).

EXAMPLES 7 and 8

The 10-amino acid region removed in the M57 deletion mutant (see Figure 1) is adjacent to but does not contain a N-linked glycosylation site. To  
30 investigate whether the region of the M57 deletion is involved directly in CD2-binding or indirectly disrupts CD2-binding by causing a conformational change in the CD2-binding domain of LFA-3, two additional experiments were performed.

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First, three large deletion mutants were generated, each of which lacked a span of 59 amino acids compared to the native LFA-3 molecule. The regions deleted are shown by underlining in Figure 6  
5 and were designated M100, M101 and M102. The object of this experiment was to determine whether the conformational changes inherent in the large M101 and M102 deletions would alter recognition of the M57 region (see Figure 1) and to demonstrate that the  
10 entire CD2 binding domain was encompassed in the M100 region.

Second, a synthetic oligopeptide, LF08, having the amino acid sequence (SEQ ID NO:7) Lys Asn Arg Val Tyr Leu Asp Thr Val Ser Gly Ser Leu Thr Ile Tyr  
15 (amino acids +50--+65 of mature LFA-3 (see Figure 1)) was isolated and fixed to a solid substrate, then used in a Jurkat cell rosetting assay to determine if CD2-expressing cells would recognize the isolated M57 region of LFA-3 (see Figure 1).

20 Preparation of Large Deletion Mutants

Figure 6 depicts the amino acid sequence and nucleotide sequence of transmembrane LFA-3 and shows with underlining the three regions, designated M100, M101 and M102, which were deleted from LFA-3 cDNA to  
25 yield genes capable of directing the expression of three large deletion mutants.

Following the procedure used in Example 1, a 30-base antisense oligonucleotide was synthesized which consisted of sequences complementary to the 15 bases of  
30 the sense strand upstream of (5' to) and to the 15 bases downstream of (3' to) the desired deletion region. The synthetic oligonucleotides used to generate the large deletion mutations are shown below:

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<u>Sequence</u>	<u>Complementary to LFA-3 Nucleotides</u>	<u>Segment Deleted</u>
SEQ ID NO:30	109-123+304-318	M100
SEQ ID NO:31	289-303+484-498	M101
5       SEQ ID NO:32	469-483+634-648	M102.

Heteroduplexing was carried out as described in Example 1, except that 200 ng of LFA-3 cDNA was used. Colony hybridizations were carried out using  $1 \times 10^6$  CPM/ml  $^{32}$ P-ATP kinased oligonucleotides at 65°C and 10 washed with 0.5x SSC at 60°C. Positives were screened by EcoRI digestion and isolation of 2900 bp and 500 bp fragments on a 0.7% TAE agarose gel.

Construction of expression vectors was performed by excising the LFA-3 cDNA from deletion 15 mutation plasmids prepared above with NotI. Aliquots of 50 µg of a eukaryotic expression plasmid PMDR902 (obtained from Biogen, Inc., Cambridge, Massachusetts) were digested with 50 units of NotI, and the isolated NotI fragments (0.06 pmol), containing the deletion 20 mutations M100, M101 and M102, were ligated to 0.02 pmol PMDR902 DNA, and the ligated products were used to transform E.coli JA221.

Positive clones were screened by colony hybridization using oligonucleotides 18, 19 and 20, 25 having the sequences of SEQ ID NO:30, SEQ ID NO:31 and SEQ ID NO:32, respectively, as probes. The orientation of the inserts was determined by PvuII restriction analysis, which generated bands of approximately 6599, 1737 and 500 bp on 20.7% TAE agarose gel for correctly 30 oriented constructs. The sequence was confirmed by DNA sequencing as described above. The resulting recombinant expression plasmids containing the M100, M101 and M102 deletion mutations were designated

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pPMDRM100-4, pPMDRM101-1 and pPMDRM102-8, respectively. The resulting expression plasmids were electroporated into CHO cells as in Example 2, except the cells were split into medium containing 25 nM and 50 nM

5 methotrexate.

The M100/CHO, M101/CHO and M102/CHO transfectants were subjected to a Jurkat cell binding assay, as described in Example 4 above. The results are shown in Figure 7. M101/CHO cells (Figure 7C) and 10 M102/CHO cells (Figure 7D), which express a surface protein having the intact M57 CD2-binding region of LFA-3, showed clumping characteristic of Jurkat/CHO cell binding. CHO control cells (Figure 7A) and M100/CHO cells (Figure 7B) showed no such clumping, 15 indicating the absence of a surface structure on the CHO cell surface recognized by Jurkat cells.

Jurkat Binding to Immobilized LF08 Peptide

1.4 mg of the synthetic peptide LF08 (SEQ ID NO:7) or control hepatitis B peptide MXC-01 20 (SEQ ID NO:4) at 2 mg/ml in deionized water was coupled to 100 µl agarose gel beads (Affi-Gel 10, BioRad, Richmond, California) according to the following protocol: 100 µl of Affi-Gel 10 slurry was transferred to a small Buchner funnel and the solvent drained. The 25 beads were washed with three bed volumes of cold (4°C) deionized water, transferred to 700 µl of the LF08 solution, and then incubated for 4 hours at 4°C on a rocker. A spectrum taken of the supernatant, before and after coupling of LF08, indicated that a coupling 30 efficiency of 80% had been achieved.

After coupling, unreacted coupling sites were blocked by incubating the LF08 beads or MXC-01 beads with ethanolamine/HCl (93 mM, pH 7.9) overnight at 4°C with gentle shaking. The blocked beads were then

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washed three times with PBS supplemented with 0.5 M NaCl and three times with PBS supplemented with 10% FCS.

Jurkat cells ( $2 \times 10^5$ ) were washed twice with  
5 RPMI, 4 mM L-glutamine, 10% PBS and combined with 2.5 or 15  $\mu$ l of LF08/Affi-Gel 10 beads or MXC-01/Affi-Gel 10 beads to a final volume of 20  $\mu$ l. The mixture was incubated for 30 or 60 minutes at room temperature. After incubation, the cells were transferred to 200  $\mu$ l  
10 RPMI in a 96-well flat bottom plate (Corning), and observed under a microscope at 40X and 100X. Figures 8A and 8B show characteristic results of contacting the LF08/Affigel beads with Jurkat cells, resulting in binding of coated beads to the surface of Jurkat cells.  
15 Figure 8C shows characteristic results of the MXC-01/Affigel beads failing to bind to the surface of Jurkat cells.

EXAMPLES 9 and 10

A further deletion mutant was prepared which  
20 provided a PI-linked surface polypeptide on CHO cells having the N-terminal 89 amino acids of native LFA-3. Referring to Figure 9, the nucleotides deleted from cDNA encoding PI-linked LFA-3 are shown by the underlined region, PIM3. The PIM3 nucleotides were  
25 deleted using the same heteroduplex deletion mutagenesis strategy described above (Example 1). In this case, a 30-base antisense oligonucleotide (oligonucleotide 320.03), having the 5'-3' sequence (SEQ ID NO:8) TAATGGATTG CTAAGAAAGA ACTTCATGGT, was  
30 used for deletion mutagenesis.

Plasmid p24 (100 ng), containing the cDNA encoding PI-linked LFA-3 was digested with NotI, and the large restriction fragment (lacking the PI-linked LFA-3 coding sequence) was isolated. Additional p24

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DNA was linearized with ScaI. The large NotI restriction fragment and the ScaI-linearized DNA were denatured, mixed with oligonucleotide 320.03, and allowed to reanneal as described in Example 1. After 5 annealing, the gaps in the heteroduplexes were filled in, and the fragments ligated as described in Example 1.

The filled-in, ligated heteroduplexes were then transformed into E.coli JA221 and the 10 transformants screened by colony hybridization using a <sup>32</sup>P-labeled 320.03 oligonucleotide ( $1 \times 10^6$  cpm/ml) at 60°C as described above. The hybridization filters were washed in 0.5X SSC at 65°C and positive colonies identified by autoradiography. Plasmid DNA was 15 isolated from each of the positive colonies, and digested with NotI. Digestion of one of the plasmids, pPIM3-6, with NotI yielded fragments of 2648 and 640 bp, confirming that pPIM3-6 contained the desired PIM3 deletion in the PI-linked LFA-3 coding sequence. 20 The 640 bp NotI fragment of pPIM3-6 was cloned into the NotI site of the expression vector PMDR902 (a gift of Dr. M.D. Rosa, Biogen, Inc., Cambridge, Massachusetts), and used to transform E.coli JA221 cells. Transformants were screened by colony 25 hybridization using the <sup>32</sup>P-labeled 320.03 oligonucleotide as probe. Plasmids from positive colonies were then isolated, and the orientation of the DNA insert determined by restriction enzyme analysis using PvuII. Recombinant plasmid pPMDRM3-10 contained 30 PvuII fragments of 6399, 1634 and 495 bp, indicating that this plasmid contained the mutated PI-linked LFA-3 gene in the proper orientation for expression in the PMDR902 vector.

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Expression of PIM3 Deletion Mutant

pPMDRM3-10 was electroporated into CHO DHFR<sup>-</sup> cells and amplified with 25 and 50 nM methotrexate as described above. Cells from plates containing

5 confluent growth were assayed by FACS using MAbs TS2/9 and 7A6 as described above. The PIM3 deletion mutant form of LFA-3 expressed in a cell line designated PIM3.25.2 exhibited epitopes involved with CD2/LFA-3 complex formation.

10 To determine whether the protein recognized by TS2/9 and 7A6 on PIM3.25.2 cells was PI-linked, the protein expressed on the surface of PIM3.25.2 cells was tested for its susceptibility to release by treatment with phosphatidylinositol-specific phospholipase C (PI-PLC, obtained from Biogen, Inc.). Cells of PIM3.25.2 were grown to 70 percent confluence in two 100 mm culture plates and removed with PBS supplemented with EDTA (5 mM) as described above. One-third of these cells was treated with PI-PLC (1 µl; Biogen, Inc.) at

15 20 37°C for 45 minutes in 100 µl of alpha<sup>-</sup> MEM supplemented with 10% FCS, L-glutamine (2 mM), pen-strep mix (1X), and 25 nM methotrexate. The cells were then centrifuged for 15 seconds in an Eppendorf microcentrifuge and washed two times in FACS buffer

20 25 30 37°C for 45 minutes in 100 µl of alpha<sup>-</sup> MEM supplemented with 10% FCS, L-glutamine (2 mM), pen-strep mix (1X), and 25 nM methotrexate. The cells were then centrifuged for 15 seconds in an Eppendorf microcentrifuge and washed two times in FACS buffer (PBS, 0.5% BSA, 0.1% sodium azide, pH 7.2). The washed cells were then resuspended in FACS buffer containing FITC-labeled goat anti-mouse IgG (1:50 dilution from serum). The cells were then washed twice with FACS buffer, resuspended in 300 µl of PBS and transferred to Falcon 2052 tubes for FACS analysis.

Referring to Figure 10, cells of PIM3.25.2 clearly express a protein on their cell surface that is recognized by TS2/9 (Figure 10A, dashed line) and is also susceptible to release from the cell surface by 35 PI-PLC treatment (Figure 10A, dotted line). Since the

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PIM3 protein expressed in cells of clone PIM3.25.2 possesses LFA-3 epitopes recognized by MAb TS2/9, which blocks CD2/LFA-3 complex formation, it was concluded that the 89 amino acid N-terminal region of the mature 5 LFA-3 molecule contains the conformational requirements necessary for CD2/LFA-3 complex formation.

To increase expression of the PIM3 mutant LFA-3 protein, the PIM3.25.2 clone was amplified in alpha<sup>-</sup> MEM supplemented with 10% FCS in 50, 100 and 200 10 nM of methotrexate. Cells were plated in 96-well microtiter plates at a concentration of 20 cells/ml, grown to confluence (12-17 days) and expanded to 100 mm plates for subsequent FACS analysis as described for PIM3.25.2, except that expression of the PIM3 mutant 15 form of LFA-3 was monitored using the 7A6 MAb (Figure 10B, dashed line). The results of the FACS analysis indicated that the amplified cell line, PIM3.25.2.100.12, expressed approximately ten-fold higher amounts of the PIM3 mutant form of PI-linked 20 LFA-3. Treatment of the PIM3.25.2.100.12 cells with PI-PLC released about 80% of the PIM3 mutant LFA-3 (Figure 10B, dotted line).

#### EXAMPLE 11

##### Construction of plasmid pSAB144

25 pSAB144 contains the human IgG1 constant region sequences excluding the C<sub>H</sub>1 region and the first cysteine of the amino terminal portion of the hinge region. The human IgG1 heavy chain constant region was isolated from a partial Sau3A human fibroblast genomic 30 library (EMBL/5X, a gift of Dr. Mark Pasek). The library DNA was cleaved with HindIII and PvuII. DNA fragments corresponding to approximately 3 kb were ligated to the vector fragment of pSP65 (Promega, Madison, Wisconsin) generated by cleavage of pSP65

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using HindIII and HincII. The resulting plasmid was designated pAB1. Plasmid pAB1 was used as a source of DNA encoding the IgG1 heavy chain constant region.

In order to isolate a cDNA copy of the IgG1 heavy chain region, RNA was prepared from COS7 cells which had been transiently transfected by the plasmid VCAM1-IgG1 (also known as pSAB133). Construction of plasmid VCAM1-IgG1 is described in PCT patent application WO 90/13300. The RNA was reverse transcribed to generate cDNA using reverse transcriptase and random hexamers as the primers. After 30 min. at 42°C, the reverse transcriptase reaction was terminated by incubation of the reaction at 95°C for 5 min. The cDNA was then amplified by PCR (Polymerase Chain Reaction, see, e.g., Sambrook et al., Molecular Cloning, Vol. 3, pp. 14.1-14.35 (Cold Spring Harbor; 1989)) using the following kinased primers:

370-31:

(SEQ ID NO:34): 5' TCGTC GAC AAA ACT CAC ACA TGC C,  
20 (SEQ ID NO:35) asp lys thr his thr cys

which contains a SalI site, and

370-32 (SEQ ID NO:36):

5' GTAAATGAGT GCGGCGGCCG CCAA,

which encodes the carboxy terminal lysine of the IgG1 heavy chain constant region, followed by a NotI site.

The PCR amplified cDNA was purified by agarose gel electrophoresis and glass bead elution for cloning in plasmid pNN03. Plasmid pNN03 was constructed by removing the synthetic polylinker sequence from the commercially available plasmid pUC8 (Pharmacia, Piscataway, New Jersey) by restriction endonuclease digestion and replacing the synthetic polylinker sequence with the following novel synthetic sequence (SEQ ID NO:37):

35 GCGGCCGCGG TCCAACCACC AATCTCAAAG CTTGGTACCC GGGAAATTCA

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ATCTGCAGCA TGCTCGAGCT CTAGATATCG ATTCCATGGA TCCTCACATC  
CCAATCCGCG GCCGC.

The purified PCR amplified cDNA fragment was ligated to pNN03 which had been cleaved with EcoRV,  
5 dephosphorylated, and purified by low melt agarose gel electrophoresis. The ligation reaction was used to transform E.coli JA221 and the resulting colonies were screened for a plasmid containing an insert of approximately 700 bp. The identity of the correct  
10 insert was confirmed by DNA sequence analysis, and the plasmid was designated pSAB144.

Construction of Plasmid pSAB149

The plasmid pSAB149 was constructed as follows. The LFA3-encoding plasmid pHT16-6 was  
15 subjected to PCR amplification using oligonucleotides 320-04 and 320-05. Oligonucleotide 320-04 includes a NotI site and the nucleotides corresponding to amino acids 1 through 7 of the LFA-3 signal sequence:  
20 (SEQ ID NO:38) 5' GAGGCCGGCCG CC ATG GTT GCT GGG AGC GAC  
GCG,  
(SEQ ID NO:39) met val ala gly ser asp  
ala.

Oligonucleotide 320-05 corresponds to the LFA-3 amino acids 86-92 and includes a SalI site (SEQ ID NO:40):  
25 5' AAGTCGACAT AAAGAAAGAA CTTCAT. The amplified DNA fragment was ligated to the vector fragment of pNN03, cleaved by EcoRV.

Construction of pSAB132

pJOD-S (Barsoum, J., DNA and Cell Biol., 2,  
30 pp. 293-300 (1990)) was modified to insert a unique NotI site downstream from the adenovirus major late promoter so that NotI fragments could be inserted into the expression vector. pJOD-S was linearized by NotI cleavage of the plasmid DNA. The protruding 5' termini

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were blunt-ended using Mung Bean nuclease, and the linearized DNA fragment was purified by low melting temperature agarose gel electrophoresis. The DNA fragment was religated using T4 DNA ligase. The 5 ligated molecules were then transformed into E.coli JA221. Colonies were screened for the absence of a NotI site. The resulting vector was designated pJOD-S delta Not1. pJOD-S delta Not1 was linearized using SalI and the 5' termini were dephosphorylated using 10 calf alkaline phosphatase. The linearized DNA fragment was purified by low melting temperature agarose gel electrophoresis and ligated in the presence of phosphorylated oligonucleotide ACE175, which has the following sequence: (SEQ ID NO:41) TCGACGCGGC CGCG. 15 The ligation mixture was transformed into E.coli JA221, and colonies were screened for the presence of a plasmid having a NotI site. The desired plasmid was named pMDR901.

In order to delete the two SV40 enhancer 20 repeats in the SV40 promoter which controls transcription of the DHFR cDNA, pMDR901 and pJODΔe-tPA (Barsoum, DNA and Cell Biol., 9, pp. 293-300 (1990)) were both cleaved with AatII and DraIII. The 2578 bp AatII-DraIII fragment from pMDR901 and the 5424 bp 25 AatII-DraIII fragment from pJODΔe-tPA were isolated by low melting temperature agarose gel electrophoresis and ligated together. Following transformation into E.coli JA221, the resulting plasmid, pMDR902, was isolated. pSAB132 was then formed by eliminating the EcoRI-NotI 30 fragment of pMDR902 containing the adenovirus major late promoter and replacing it with an 839 bp EcoRI-NotI fragment from plasmid pCMV-B (Clontech, Palo Alto, California) containing the human cytomegalovirus immediate early promoter and enhancer.

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Construction of pSAB152

pSAB144 was cleaved with SalI and NotI, and the 693 bp fragment isolated. pSAB149 was cleaved with SalI and NotI and the 365 bp fragment was isolated.

- 5 The two fragments were ligated to pSAB132, which had been cleaved with NotI, and the 5' termini dephosphorylated by calf alkaline phosphatase. The resulting plasmid, pSAB152, contained the DNA sequence encoding the LFA-3 signal sequence, the amino terminal  
10 92 amino acids of mature LFA-3, ten amino acids of the hinge region of IgG1 and the C<sub>H</sub>2 and C<sub>H</sub>3 constant domains of IgG1 (see Figure 12). E.coli JA221 transformed with pSAB152 is deposited with American Type Culture Collection, Rockville, Maryland (accession  
15 no. 68720).

Construction of pNNM57-14

As mentioned in Example 1, plasmid pLFA3M57-4A contains a DNA sequence encoding LFA-3 with a deletion of the 30 bp M57 region (i.e., nucleotides  
20 244-273 of SEQ ID NO:9). This coding sequence of pLFA3M57-4A was subjected to PCR amplification using oligonucleotide 320-04 (SEQ ID NO:38) and oligonucleotide 320-05 (SEQ ID NO:40) in order to amplify the DNA encoding the signal sequence and the  
25 first 82 amino acids of the M57 deletion mutant of LFA-3. The PCR amplified DNA was purified by low melting temperature agarose gel electrophoresis.

The purified, PCR amplified DNA was then ligated into the EcoRV site of plasmid pNN03. The  
30 ligated DNA was transformed into E.coli JA221, and the resulting transformants screened (by NotI digestion) for a plasmid containing an approximately 300 bp insert. The identity of the desired insert was confirmed by DNA sequence analysis. The desired

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plasmid containing the approximately 300 bp insert was designated pNNM57-14.

Construction of pM57Ig-5

Plasmid pNNM57-14 was cleaved with NotI and 5 SalI, and the 310 bp fragment isolated by low melting temperature agarose gel electrophoresis. This fragment and the 693 bp SalI-NotI fragment of pSAB144 (see supra) were ligated to NotI-digested, calf alkaline phosphatase-treated pSAB132 (see supra). The ligation 10 mixture was used to transform E.coli JA221, and transformant colonies were screened by hybridization using a  $\gamma$ -<sup>32</sup>P-ATP labeled oligonucleotide having the sequence of SEQ ID NO:17. The desired orientation of the inserts in pSAB132 was confirmed by the 15 identification of a 6913 and a 2029 bp fragment upon digestion with PvuII. The resulting plasmid was designated pM57Ig-5. DNA sequence analysis confirmed that pM57Ig-5 contains a DNA sequence encoding the LFA- 20 3 signal sequence, the first 82 amino acids of the M57 deletion mutant of LFA-3, ten amino acids of the hinge region of human IgG1 and the C<sub>H</sub>2 and C<sub>H</sub>3 constant domains of IgG1.

EXAMPLE 12

25 Production of LFA3TIP From  
Transiently Transfected COS7 Cells

To produce the LFA-3-Ig fusion protein, LFA3TIP (also referred to as LFA-3(92)IgG), plasmid pSAB152 DNA was transfected into COS7 cells by electroporation as follows. Four aliquots of 200  $\mu$ g 30 each of pSAB152 DNA each were ethanol precipitated with 350  $\mu$ g of sonicated salmon sperm DNA. DNA was pelleted and resuspended in 0.8 ml of 1 x HEBS (20 mM Hepes/pH 7.05, 137 mM NaCl, 5 mM KCl, 0.7 mM Na<sub>2</sub>HPO<sub>4</sub>, 6 mM

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dextrose). COS7 cells from 4 confluent T150 flasks ( $8 \times 10^7$  cells) were removed by trypsin treatment in DMEM (Gibco, Gaithersburg, Maryland), 10% fetal bovine serum, 4 mM glutamine. Cells from each flask were  
5 transferred to individual 15 ml Corning polypropylene tubes and pelleted at 1000 rpm for 4 minutes at room temperature in an IEC centrifuge. Medium was aspirated, and cells resuspended in the DNA-HEBS solution and transferred to a Bio-Rad (Richmond, California) electroporation cuvette. The cuvettes were set in a Bio-Rad Gene Pulser and pulsed at 280 volts and 960  $\mu$ Fd capacitance. Cells were resuspended in 10 ml of DMEM medium and pelleted in an IEC centrifuge as described above. All cells were seeded into a 2  
10 liter cell factory in DMEM medium, and culture medium was harvested after 72 hours for purification of  
15 LFA3TIP protein.

The concentration of secreted LFA3TIP in transfected COS7 culture medium was determined by  
20 ELISA. Fisher 96 flat bottom well plates (Corning 2580) were coated with 50  $\mu$ l of goat anti-human IgG (H+L) (Jackson Immuno Research, West Grove, Pennsylvania, Catalogue No. 109-005-088) diluted at 5 $\mu$ g/ml in 1X PBS. These plates were incubated at 4°C  
25 overnight. Plates were washed the next day 6 times with dH<sub>2</sub>O and blocked with 150  $\mu$ l/well of block buffer (2% normal goat serum in 1X PBS). Buffer was removed after a 2 hour incubation at room temperature and conditioned medium was added at various dilutions. As  
30 a control to create a standard curve, a series of dilutions of whole human IgG (Jackson Immuno Research, West Grove, Pennsylvania, Catalogue No. 099-000-603) was included on the same plate. After a one hour incubation at room temperature, plates were washed 4  
35 times with 0.01% Tween 20 in 1X PBS, and 50  $\mu$ l/well of

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a 1:5000 serum dilution of alkaline phosphatase-conjugated anti-human IgG specific for Fc fragment (Jackson Immuno Research, West Grove, Pennsylvania, Catalogue No. 109-055-098) was added to each well.

5 After one hour, plates were washed 6 times with 0.1% Tween 20.

Plates were developed with a solution containing a 1:10 dilution of 10 mM Ca<sup>2+</sup>, Mg<sup>2+</sup> in 10% diethanolamine, pH 9.6, plus 5 mg/ml 4-

10 nitrophenylphosphate (Boehringer Mannheim Biochemicals, Indianapolis, Indiana, catalogue no. 738-352). The reaction was stopped using 3N NaOH and plates read at 405 nm on a Microdevices microplate reader.

15 Production Of LFA3TIP From A Stably Transformed CHO Cell Line

A recombinant LFA3TIP expression vector was constructed as described below, which can be stably maintained in CHO cells to achieve continuous expression of LFA3TIP.

20 A NotI fragment containing the LFA3TIP coding sequence of pSAB152 was purified by low melting temperature agarose gel electrophoresis. The fragment was ligated into the NotI cloning site of the expression plasmid pMDR902 (see Example 11). The 25 resulting ligations were used to transform E.coli JA221, and colonies containing the desired, correctly oriented, insert were identified by the presence of 6599, 2058, and 487 bp fragments upon digestion with PvuII. The identity of the correct insert in pMDR902 30 was confirmed by DNA sequence analysis. The resulting recombinant expression plasmid was designated pMDR(92)Ig-3.

The recombinant expression plasmid pMDR(92)Ig-3 was electroporated into CHO cells

35 according to the published protocol of J. Barsoum (DNA

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Cell Biol., 9, pp. 293-300 (1990)), with the following changes. 50 µg of AatII-digested plasmid DNA and 350 µg of sonicated salmon sperm DNA were used in the electroporation protocol. In addition, cells were

5 selected in alpha<sup>-</sup> complete medium supplemented with 25 or 50 nM methotrexate (MTX).

To determine expression levels of secreted LFA3TIP, clones were transferred to a flat bottom 96 well microtiter plate, grown to confluence and assayed

10 by ELISA (see infra). One clone grown in the presence of 25 nM MTX, LFA3TIP.25.11, expressed 5-10 µg of LFA3TIP per ml of culture medium. This clone was expanded and amplified further. Amplification was carried out by seeding 1 cell per well in a 96 well

15 plate with complete medium supplemented with 50 or 100 nM MTX. Clones were grown to confluence and assayed by ELISA. Two clones, LFA3TIP.25.11.50.10A and LFA3TIP.25.11.50.5B, secreted higher levels (i.e., 50-60 µg of LFA3TIP per ml of culture medium) of LFA3TIP

20 and were expanded for further study and purification.

The concentration of secreted LFA3TIP in medium of cultures of CHO cells carrying pMDR(92)Ig-3 (i.e., conditioned medium) was determined by ELISA.

Wells of Immulon 2 plates (Dynatech,  
25 Chantilly, Virginia) were each coated with anti-LFA3 MAb TS2/9 (gift of Dr. Timothy Springer) with 50 µl of anti-LFA3 TS2/9 MAb diluted to 25 µg/ml in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6, covered with Parafilm, and incubated overnight at room  
30 temperature. The next day, plates were washed 6 times with deionized water and blocked with 150 µl/well of a block buffer (5% fetal calf serum in 1X PBS), which had been filtered through a 2 micron filter. The buffer was removed after a 2 hour incubation at room  
35 temperature, and conditioned medium was added at

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various dilutions. As a control to create a standard curve, a series of dilutions of LFA-3 (50  $\mu$ l per well) was also included. Typically, the well containing the most concentrated LFA-3 standard contained 50  $\mu$ l of an 5 LFA-3 solution containing 10 ng of LFA-3 in 1X PBS. Block buffer diluted in 1X PBS constituted the negative control. The samples and controls were incubated at room temperature for one hour.

The plates were then washed 6 times with 10 deionized water. Each well was then filled with 50  $\mu$ l of a 1:5000 dilution of rabbit polyclonal anti-LFA-3 antiserum (e.g., antiserum 202, supra) in 5% fetal calf serum in 1X PBS. After one hour at room temperature, the polyclonal anti-LFA-3 antiserum was removed and the 15 wells were washed 4 times with a solution of 0.05% Tween-20 in 1X PBS. Each well was then filled with 50  $\mu$ l of HRP-goat anti-rabbit IgG(H+L) (Jackson Immuno Research Laboratories, Inc., West Grove, Pennsylvania; Catalogue No. 111-035-045) at a 1:10,000 dilution in 20 the block buffer containing 2% whole mouse serum (Cappel, Catalogue No. 5011-1380). The plates were then incubated at room temperature for 50-60 minutes.

The HRP-goat anti-rabbit IgG(H+L) solution was removed, and the wells were washed 6 times with 25 0.05% Tween-20 in 1X PBS. Then, 50  $\mu$ l of HRP-substrate buffer was added to each well at room temperature. HRP-substrate buffer was prepared as follows: 0.5 ml of 42 mM 3,3',5,5'-tetramethylbenzidine (TMB), (ICN Immunobiologicals, Lisle, South Carolina, Catalogue 30 No. 980501) in DMSO (Aldrich) was slowly added to 50 ml of substrate buffer (0.1 M sodium acetate/citric acid, pH 4.9); followed by addition of 7.3  $\mu$ l of 30% hydrogen peroxide (Sigma, Catalogue No. H-1009).

The development of a blue color in each well 35 was monitored at 650 nm on a microtiter plate reader.

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After 7-10 minutes, the development was stopped by the addition of 50  $\mu$ l of 2 N sulfuric acid. The resulting yellow color was read at 450 nm on a microtiter plate reader. A negative control well was used to blank the  
5 machine.

Production of M57IgG

A fusion protein consisting of LFA3TIP lacking the ten amino acid M57 region of LFA-3 (M57IgG) was produced substantially as described above for  
10 LFA3TIP.

EXAMPLE 13

Purification of LFA3TIP

The COS7(pSAB152) conditioned culture medium was concentrated 10-fold using an AMICON S1Y30 spiral  
15 cartridge system (AMICON, Danvers, Massachusetts). The concentrate was divided into 4 x 50 ml aliquots and batch-adsorbed with 200  $\mu$ l per aliquot of Protein-A Sepharose 4B (SIGMA, St. Louis, Missouri) overnight at 4°C. The beads were pelleted at 1000 rpm in a clinical  
20 centrifuge, combined, and then packed into a 5 mm diameter column. The resin was washed with 20 ml of PBS supplemented with 500 mM NaCl followed by 20 ml of PBS. The bound proteins were eluted in 150  $\mu$ l fractions with 50 mM glycine, 250 mM NaCl (pH 3.0) into  
25 tubes containing 15  $\mu$ l of 1 M HEPES, pH 7.8. 10  $\mu$ l of each fraction was run on 12% nonreducing SDS-PAGE gels. The fractions containing the eluted protein were pooled and subjected to Superose-12 gel filtration chromatography developed in PBS (Pharmacia/LKB,  
30 Piscataway, New Jersey).

Aliquots of 10  $\mu$ l of the peak protein fractions (determined by measuring the O.D. at 280 nm) were run on 12% SDS-PAGE gels under nonreducing

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conditions and the fractions containing LFA3TIP with a low concentration of contaminating proteins, as determined on the SDS-PAGE gels and by Western blot analysis (see, e.g., Towbin et al., Proc. Natl. Acad.

5 Sci. USA, 74, pp. 4350-54 (1979); Antibodies: A Laboratory Manual, pp. 474-510 (Cold Spring Harbor Laboratory, 1988)), were pooled and concentrated in a YM30 Centricon (AMICON, Danvers, Massachusetts). LFA-3 was detected on Western blots using rabbit anti-LFA-3  
10 polyclonal antiserum 202 (see Example 3, supra) and goat anti-rabbit IgG, which was labeled with horseradish peroxidase (Sigma, St. Louis, Missouri). Blots were developed using the Amersham ECL detection kit (RPN 2106, Amersham, Arlington Heights, Illinois).  
15 The final pool was run on 12% SDS-PAGE gels under nonreducing conditions to assess the purity. A UV absorbance spectrum was run to determine the concentration. All preparations purified with Protein A were found to contain a single contaminant, which  
20 most likely is bovine IgG, present in the fetal calf serum of the culture medium, and which copurifies with the LFA3TIP.

The purity of LFA3TIP was determined by denaturing and nondenaturing SDS-PAGE (Figure 13). The  
25 results of the SDS-PAGE analysis indicated that LFA3TIP, purified from the culture medium of COS7 cells carrying plasmid pSAB152, is present in the cell culture medium as a dimer of two monomeric LFA-3-Ig fusion proteins, connected by disulfide bonds.

30 The fact that LFA3TIP is found in the cell culture medium suggests that, as with LFA-3, the signal peptide sequence operates normally, i.e., the signal peptide is cleaved from the amino terminal region of the LFA-3 domain of LFA3TIP in the process of secreting  
35 LFA3TIP protein across the cell membrane.

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Purification of M57IgG

M57IgG was purified from conditioned culture medium substantially as described above for LFA3TIP. Similar contaminants were observed.

5

EXAMPLE 14

FACS Analysis of LFA3TIP Binding

Jurkat cells were transferred into several Eppendorf tubes at  $1 \times 10^5$  cells/tube. Cells were pelleted for 10 seconds in an Eppendorf

- 10 microcentrifuge, the medium was aspirated off, and pellets were resuspended in 100  $\mu$ l of LFA3TIP or anti-CD2 monoclonal antibody (MAb) TS2/18 (Sanchez-Madrid et al., *Proc. Natl. Acad. Sci. USA*, **79**, pp. 7489-7493 (1982)), both at 10  $\mu$ g/ml in FACS buffer (PBS, 0.1% NaN<sub>3</sub>, 0.5% BSA pH 7.2). Cells were incubated on ice for 30 minutes, washed twice with FACS buffer, and incubated with 100  $\mu$ l of the appropriate secondary antibody to detect MAbs bound to the Jurkat cells.
- 15 FITC conjugated goat anti-mouse IgG (H+L) F(ab')<sub>2</sub> (Jackson Immunoresearch) was used to detect the anti-CD2 MAb, TS2/18, and R-Phycoerythrin conjugated AP goat anti-human IgG F(ab')<sub>2</sub> (Jackson Immunoresearch) was used to detect LFA3TIP bound to Jurkat cells. The FITC goat anti-mouse IgG F(ab')<sub>2</sub> was diluted to a 1:50
- 20 concentration in FACS buffer, and the R-Phycoerythrin conjugated AP goat anti-human IgG F(ab')<sub>2</sub> was diluted by 1:20 in FACS buffer. Cells were incubated for 30 minutes on ice, washed 2 times and resuspended in 300  $\mu$ l of 1 x PBS and fluorescence detected on a cell
- 25 sorter.
- 30

For competition studies, Jurkat cells were pelleted in Eppendorf tubes as described above and

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LFA3TIP was added at a concentration of 10 µg/ml in FACS buffer. Cells were incubated for 30 minutes on ice and washed twice with FACS buffer. Pellets were then resuspended in 100 µl of a 1:100 dilution (in FACS buffer) of anti-CD2 MAb-containing ascites fluids:  
5 T11<sub>1</sub>, T11<sub>2</sub> or T11<sub>3</sub> (all were gifts of Dr. Ellis Reinherz, Dana Farber Cancer Institute, Boston, Massachusetts). Cells were incubated for 30 minutes on ice, washed twice and resuspended in a 1:20 dilution of  
10 R-phycoerythrin conjugated AP goat anti-human IgG F(ab')<sub>2</sub> to detect bound LFA3TIP. After a 30 minute incubation on ice, cells were washed twice and resuspended in 300 µl of 1 x PBS and analyzed in a cell sorter.  
15 The TS2/18 monoclonal antibodies and the monoclonal antibodies in ascites fluid T11<sub>1</sub> are specific for the LFA-3 binding domain of CD2. Thus, these antibodies are expected to bind to CD2 molecules on the surface of Jurkat cells and occupy the same site  
20 at which LFA3TIP binds. Accordingly, when Jurkat cells were incubated first with an excess of TS2/18 MAb or T11<sub>1</sub> ascites fluid, few if any domains are expected to be available for R-Phycoerythrin conjugated LFA3TIP to molecules to bind. As shown in Figure 14A,  
25 preincubation of Jurkat cells with either the TS2/18 MAb or T11<sub>1</sub> ascites fluid resulted in few cells being labeled with LFA3TIP. A signal nearly identical to unstained control cells was observed. In contrast, in the absence of either TS2/18 or T11<sub>1</sub> ascites fluid,  
30 LFA3TIP bound to Jurkat cells and labeled a significant proportion of the cells (Figure 14A).  
The MAbs present in T11<sub>2</sub> and T11<sub>3</sub> ascites fluid are specific for CD2, but recognize epitopes distinct from the epitope of CD2 involved in CD2/LFA-3  
35 complex formation. As shown in Figure 14B,

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preincubation of Jurkat cells with either T11<sub>2</sub> or T11<sub>3</sub> ascites fluid did not prevent LFA3TIP from binding to the Jurkat cells. The results shown in Figure 14A and 14B indicate that LFA3TIP possesses the functional CD2-5 binding domain LFA-3.

EXAMPLE 15

Mixed Lymphocyte Reaction

A functional assay for the formation of the CD2/LFA-3 complex and T-cell activation is the mixed lymphocyte reaction ("MLR") (see, e.g., Krensky et al., 10 *J. Immunol.*, 131(2), pp. 611-616 (1983); Bradley, "Mixed Lymphocyte Responses", in Selected Methods in Cellular Immunology (Mishell and Shiigi, eds.), 15 pp. 162-164 (W.H. Freeman & Co., San Francisco 1980). This assay is based on the activation of T-lymphocytes in a population of peripheral blood lymphocytes ("PBLs") when the T-lymphocytes ("responder cells") recognize alloantigens in nonproliferating allogenic PBLs ("stimulator cells"). Such activation occurs due 20 to cell to cell adhesion, mediated in part by the binding of CD2 molecules on the T-lymphocytes to the LFA-3 molecules on the allogeneic PBLs.

PBLs were purified from 30 ml blood of two allogeneic human donors over a Ficoll-Paque gradient 25 (Pharmacia, Piscataway, New Jersey, catalog no. 17-0840-02). Blood was diluted at a 1:2 ratio in RPMI medium and overlayed on a Ficoll gradient at a 2:1 ratio (30 ml blood:15 ml Ficoll). Cells were centrifuged through the gradient in a Sorvall RT6000 30 centrifuge at 1600 rpm, 20°C, for 30 minutes. The interface containing PBLs was collected into 50 ml polypropylene centrifuge tubes (Corning 25331) and topped with RPMI medium. The cells were then pelleted by centrifugation in a Sorvall RT6000 centrifuge at

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1400 rpm, 4°C, for 15 minutes. The cells of the pellets were washed twice with 50 ml RPMI and pelleted as described above. PBLs were resuspended in RPMI complete medium (RPMI, 10% Hyclone FCS, 4 mM glutamine, 5 Pen-Strep) to a concentration of  $3 \times 10^6$  cells/ml. To produce stimulator cells, PBLs from one of the donors were irradiated at 2000 rads in a Gammacell Irradiator and brought to a final concentration of  $3 \times 10^6$  cells/ml.

10 Antibodies or LFA3TIP samples were added at the concentrations indicated in Figure 15 to a polystyrene 96 round bottom well plate (Corning 25850). The highest concentration used for both the MAbs and LFA3TIP was 5 µg/ml. All dilutions and incubations 15 were done in RPMI complete medium. Responder cells (nonirradiated) and irradiated stimulator cells were added to wells at a 1:1 ratio at a final concentration of  $1.5 \times 10^5$  cells per well each and plates were incubated for five days at 37°C. On the fifth day, 20 cells were pulsed with 1 µCi [ $\text{methyl-}^3\text{H}$ ] thymidine (New England Nuclear, Boston, Massachusetts, NET-027) for fifteen hours, and harvested on a Tomtech 96 well harvester. Proliferation of the nonirradiated population of T-lymphocytes was measured by determining 25 incorporation of the  $^3\text{H}$ -thymidine into the cells, indicating uptake of the labeled thymidine during T-lymphocyte proliferation. Using this MLR assay, a variety of MAbs and CD2-binding molecules were tested for their effect on CD2/LFA-3 complex formation and 30 T-cell activation.

The anti-LFA-3 MAb 1E6 (produced by hybridoma 1E6-2C12, ATCC accession no. HB 10693) is specific for the CD2-binding domain of LFA-3. As shown in Figure 15, when 1E6 MAbs were present in the MLR assay, 35 a dose dependent inhibition of T-cell activation was

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observed. This result is consistent with the view that 1E6 MAbs bind the CD2-binding domain of LFA-3, prevent CD2/LFA-3 complexes from forming between stimulator and responder cells, and thereby inhibit activation of

5 T-cells in the responder cell population.

LFA3TIP exhibited a similar dose dependent inhibition of T-cell activation in the MLR assay (see Figure 15). To exclude the possibility that the IgG portion of the 1E6 MAbs or LFA3TIP contributed to the  
10 observed inhibition of T-cell activation, non-specific human IgG1 (Pierce, Rockford, IL) was tested in the MLR assay. As shown in Figure 15, the nonspecific human IgG did not exhibit the dose dependent inhibition of T-cell activation seen with LFA3TIP or the 1E6 MAbs.

15 Taken together, these results demonstrate that the inhibitory activity of LFA3TIP on T-cell activation in the MLR assay resides in the LFA-3 domain and not the IgG domain of the fusion protein.

20 Inhibitory Activity on T-cell Activation of Other Molecules

In addition to LFA3TIP and 1E6 MAbs, the following molecules were also tested for inhibitory activity on T-cell activation in the MLR assay: 7A6 MAb (MAb specific for the CD2-binding domain of LFA-3,  
25 produced by hybridoma 7A6-2E5, ATCC accession no. HB 10695), TS2/18 (anti-CD2 MAb, Sanchez-Madrid et al., supra), hIgG (nonspecific total human IgG1), PI-LFA3 (multimeric PI-linked LFA-3 formed by intermolecular hydrophobic interaction at the PI anchor region of each  
30 PI-LFA-3 monomer), and a CD4-IgG1 fusion protein (analogous to LFA3TIP, but containing an amino terminal region consisting of a portion of CD4, see, e.g., PCT application WO 89/01940).

A comparison of the inhibitory activity of  
35 each of these molecules is shown in the bar graph of

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Figure 16. "LFA3IgGA" and "LFA3IgG72A" in Figure 16 are preparations of LFA3TIP differing in purity, i.e., 75% and 50% respectively. As a control, a "mock" preparation, purified from COS7 cells transfected with 5 vector DNA alone (i.e., no DNA insert encoding LFA3TIP), was added to show that the inhibitory activity on T-cell activation was not derived from a nonspecific inhibitor contained in conditioned COS7 cell culture medium. The mock preparation contains the 10 contaminant which, as discussed in Example 13, copurifies with LFA3TIP and probably is bovine IgG present in the fetal calf serum of the growth medium. All MLR assays were carried out as described above, except that each preparation of molecules was assayed 15 at a concentration of 0.1 µg protein/ml.

MAbs which recognize the CD2-binding domain of LFA-3 (7A6 and 1E6) or the LFA-3 binding domain of CD2 (TS2/18) exhibited significant inhibitory activity on T-cell activation. The ability of LFA3TIP to 20 inhibit T-cell activation increased with purity (compare LFA3IgG72A, 50% purity, with LFA3IgGA, 75% purity). Nonspecific human IgG1 and the CD4-IgG1 fusion protein failed to inhibit T-cell activation.

The multimeric form of PI-linked LFA-3 also 25 failed to inhibit T-cell activation. Despite the plurality of CD2-binding sites in multimeric PI-linked LFA-3, it appears that this type of multimer neither inhibits nor enhances the human allogeneic MLR.

#### EXAMPLE 16

##### 30 PBL Proliferation Assay

Human PBLs were isolated from 20 ml of human donor blood on a Ficoll-Paque gradient, as described above. Purified LFA3TIP was added to 96 well polystyrene round bottom tissue culture plates (Corning

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25850) at the concentrations indicated in Figure 17. PBLs ( $1 \times 10^5$ ) were then added to each well, and plates were incubated for 3 days at 37°C. All dilutions were carried out in RPMI complete medium (see Example 15).

5 Cells were pulsed after 3 days with 1  $\mu$ Ci/well [ $^{3}\text{H}$ ] thymidine for 15 hours and harvested on a Tomtech 96 well automatic harvester. As controls, PBL proliferation was measured in growth medium alone and in growth medium supplemented with nonspecific human

10 IgG1.

As shown in Figure 17, the LFA3TIP preparation inhibited proliferation of PBLs. Nonspecific human IgG1 did not inhibit PBL proliferation.

15 We also assayed the major contaminant in the LFA3TIP preparation from a mock preparation as described in Example 15. As mentioned in Examples 13 and 15 above, this contaminant is probably bovine IgG from the growth medium. Figure 17 shows that, as with

20 the nonspecific human IgG1, the contaminant also did not inhibit proliferation of PBLs.

PHA and OKT3 Dependent PBL Proliferation

We next assessed the ability of LFA3TIP to inhibit OKT3 dependent (i.e., anti-CD3 dependent)

25 T-cell proliferation. PBLs were isolated as described above from one healthy human donor. For OKT3 dependent PBL proliferation, aliquots of  $1 \times 10^5$  donor PBLs were incubated for 2 days with 3 ng/ml of OKT3 (Ortho Pharmaceuticals, Raritan, New Jersey) alone, and in the

30 presence of LFA3TIP (1 nM and 10 nM) or purified human IgG1 (Pierce Chemical Co., Rockford, Illinois) (1 nM and 10 nM). One aliquot of cells was incubated in medium alone, without OKT3. The cells were then pulsed

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with [ $\text{methyl-}^3\text{H}$ ] thymidine as described above. The results of this experiment are shown in Figure 18.

The results in Figure 18 show that LFA3TIP was significantly more effective at inhibiting OKT3

5 dependent PBL proliferation than the control human IgG1 of irrelevant specificity. As with the results in Figure 17, this indicates that the ability of LFA3TIP to inhibit PBL proliferation resides primarily in the 92 amino acid LFA-3 region, which contains the CD2  
10 binding domain, and not in the IgG portion of LFA3TIP.

We also assessed the ability of LFA3TIP and various other CD2-binding molecules, as well as, the ability of M57IgG, to inhibit phytohemagglutinin (PHA) dependent PBL proliferation, at both suboptimal and  
15 optimal concentrations of PHA.

For this assay,  $1 \times 10^5$  donor cells (human PBLs) were incubated with PHA (Fisher) at 0.1 or 1.0  $\mu\text{g}/\text{ml}$ , either alone or in the presence of PI-linked LFA-3 ("PILFA-3", Wallner et al., PCT patent  
20 application WO 90/02181), monomeric soluble LFA-3 ("mon LFA-3", consisting of amino acids 29-181 of SEQ ID NO:10), LFA3TIP, M57IgG (see Example 11), a full-length soluble LFA-3-IgG fusion protein ("FLIgG", consisting of amino acids 29-181 of SEQ ID NO:10 fused  
25 to a portion of the hinge region and the  $C_H^2$  and  $C_H^3$  domains of human IgG1), an anti-CD2 MAb (TS2/18, gift of T. Springer), or an anti-LFA-3 MAb (1E6) produced by hybridoma 1E6-2C12 (ATCC HB 10693). FLIgG was at a concentration of 2  $\mu\text{g}/\text{ml}$ ; all the other proteins were  
30 at a concentration of 5  $\mu\text{g}/\text{ml}$ . The cells were then pulsed with [ $\text{methyl-}^3\text{H}$ ] thymidine as described above. The results are displayed in Figure 19.

Referring to Figure 19, when PHA was incubated with human PBLs at a suboptimal concentration  
35 (i.e., 0.1  $\mu\text{g}/\text{ml}$ ), only PI-linked LFA-3 (PILFA-3) was

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able to stimulate PBL proliferation. M57IgG, monomeric soluble LFA-3 and FLIgG had no effect on suboptimal PHA-stimulated T-cell proliferation. MAb 1E6 and MAb TS2/18 inhibited proliferation 80-90%; LFA3TIP

5 inhibited 70%.

When PHA was present at an optimal level to stimulate PBL proliferation, LFA3TIP, MAb 1E6 and MAb TS2/18 inhibited T-cell proliferation by 41%, 26% and 20%, respectively.

10 The results in Figure 19 indicate that the region of LFA-3 defined by the N-terminal 92 amino acids, containing the CD2-binding domain of LFA-3, is capable of inhibiting PHA dependent PBL proliferation, but that the absence of a portion of the CD2-binding  
15 domain, as in M57IgG, or the presence of additional LFA-3 amino acid sequence, as in PI-linked LFA-3, monomeric LFA-3 or FLIgG, diminishes the ability of the CD2-binding domain of LFA-3 to inhibit PHA dependent PBL proliferation.

20 Taken together, the results indicated that inhibition of PBL proliferation by LFA3TIP is due to its LFA-3 domain.

It will be recognized from the foregoing description that a wide variety of CD2-binding  
25 polypeptides in addition to those specifically mentioned, as well as fragments and analogues of such polypeptides, will be useful additional embodiments of the present invention. All such embodiments, and other embodiments that are obvious in view of the teachings  
30 herein, are specifically contemplated and are included within the scope of the invention as defined in the following claims.

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Deposits

Host cells harboring plasmids bearing DNA sequences according to this invention were deposited under the Budapest Treaty with American Type Culture

5 Collection (ATCC), Rockville, Maryland (USA) on March 5, 1991. The deposited cultures are identified as follows:

			<u>Description:</u> (plasmid contains DNA for:)
10	<u>plasmid</u>	<u>host strain</u>	<u>Accession No.</u>
	pPYM57	<u>E.coli</u> JA221	68545 M57 deletion mutant
15	pPMDRM54-6	<u>E.coli</u> JA221	68542 M54 deletion mutant
	pPMDRM55-9	<u>E.coli</u> JA221	68546 M55 deletion mutant
	pPMDRM56-C	<u>E.coli</u> JA221	68551 M56 deletion mutant
20	pPMDRM58-15	<u>E.coli</u> JA221	68547 M58 deletion mutant
	pPYM63-4	<u>E.coli</u> JA221	68544 M63 deletion mutant
25	pPYM65-8	<u>E.coli</u> JA221	68552 M65 deletion mutant
	pPMDRM100-4	<u>E.coli</u> JA221	68550 M100 deletion mutant
	pPMDRM101-1	<u>E.coli</u> JA221	68543 M101 deletion mutant
30	pPMDRM102-8	<u>E.coli</u> JA221	68548 M102 deletion mutant
	pPMDRM3-10	<u>E.coli</u> JA221	68549 PIM3 deletion mutant

A host cell harboring a plasmid according to this 35 invention was deposited under the Budapest Treaty with the ATCC on October 1, 1991 is identified as follows:

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	<u>plasmid</u>	<u>host strain</u>	<u>Accession No.</u>	<u>Description:</u> (plasmid contains)
5	pSAB152	E.coli JA221	68720	Hybrid DNA sequence encoding LFA-3 signal sequence, N-terminal 92 amino acids of LFA-3, 10 amino acids of IgG1 hinge region, C <sub>H</sub> 2 and C <sub>H</sub> 3 IgG1 constant domains.
10				
15		A bacteriophage and a bacterial strain carrying a vector referred to herein were deposited under the Budapest Treaty with In Vitro International, Inc., Linthicum, Maryland (USA) on May 28, 1987 and May 24, 1988, respectively, and assigned accession		
20		numbers IVI-10133 and IVI-10170, respectively. These deposits were transferred to the ATCC on June 20, 1991. The deposits are identified as:		

	<u>Designation</u>	<u>Accession No.</u>	<u>Description</u>
25	λHT16[λgt10/LFA-3]	75107	Contain DNA encoding full length transmembrane LFA-3
	E.coli, BG8	68791	

The following hybridoma cell lines referred  
30 to herein were deposited under the Budapest Treaty with the ATCC on March 5, 1991:

	<u>Hybridoma</u>	<u>Accession No.</u>	<u>Antibody</u>
	7A6-2E5	HB 10695	7A6
	1E6-2C12	HB 10693	1E6

35 While we have hereinbefore described a number of embodiments of this invention, it is apparent that our basic embodiments can be altered to provide other embodiments that utilize the compositions and processes of this invention. Therefore, it will be appreciated

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that the scope of this invention includes all alternative embodiments and variations which are defined in the foregoing specification and by the claims appended hereto; and the invention is not to be  
5 limited by the specific embodiments that have been presented herein by way of example.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: BIOGEN, INC.  
WALLNER, Barbara P.  
MILLER, Glenn T.  
ROSA, Margaret D.

(ii) TITLE OF INVENTION: CD2-BINDING DOMAIN OF LYMPHOCYTE  
FUNCTION ASSOCIATED ANTIGEN 3

(iii) NUMBER OF SEQUENCES: 43

(iv) CORRESPONDENCE ADDRESS:  
(A) ADDRESSEE: Fish & Neave  
(B) STREET: 875 Third Avenue  
(C) CITY: New York  
(D) STATE: New York  
(E) COUNTRY: U.S.A.  
(F) ZIP: 10022-6250

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/667,971  
(B) FILING DATE: 12-MAR-1991

(viii) PRIOR APPLICATION DATA:

(A) APPLICATION NUMBER: US 07/770,967  
(B) FILING DATE: 07-OCT-1991

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(C) TELEX: 14-8367

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Asn Arg Val Tyr Leu Asp Thr Val Ser Gly  
1                   5                   10

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 50 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His  
1               5                   10                   15

Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys  
20              25                   30

Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser  
35              40                   45

Phe Lys  
50

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 10 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

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(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser  
1               5               10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Thr Lys Pro Asp Leu Val Asp Lys Gly Thr Glu Asp Lys Val Val Asp  
1               5               10               15  
  
Val Val Arg Asn  
20

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 77 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Val Ala Gly Ser Asp Ala Gly Arg Ala Leu Gly Val Leu Ser Val Val  
1               5               10               15  
  
Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe Ser Gln Gln Ile  
20               25               30  
  
Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val  
35               40               45

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Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu  
50 55 60

Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys  
65 70 75

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AATAGGGTTT ATTTAGACAC TGTGTCAGGT

30

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Lys Asn Arg Val Tyr Leu Asp Thr Val Ser Gly Ser Leu Thr Ile Tyr  
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TAATGGATTG CTAAGAAAAGA ACTTCATGGT

30

## (2) INFORMATION FOR SEQ ID NO:9:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1040 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 10..759

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGACGGAGCC ATG GTT GCT GGG AGC GAC GCG GGG CGG CCC CTG GGG GTC Met Val Ala Gly Ser Asp Ala Gly Arg Ala Leu Gly Val	48
1 5 10	
CTC AGC GTG GTC TGC CTG CTG CAC TCC TTT GGT TTC ATC AGC TGT TTT Leu Ser Val Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe	96
15 20 25	
TCC CAA CAA ATA TAT GGT GTG TAT GGG AAT GTA ACT TTC CAT GTA Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val	144
30 35 40 45	
CCA AGC AAT GTG CCT TTA AAA GAG GTC CTA TGG AAA AAA CAA AAG GAT Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp	192
50 55 60	
AAA GTT GCA GAA CTG GAA AAT TCT GAA TTC AGA GCT TTC TGA TCT TTT Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe	240
65 70 75	
AAA AAT AGG GTT TAT TTA GAC ACT GTG TCA GGT AGC CTC ACT ATC TAC Lys Asn Arg Val Tyr Leu Asp Thr Val Ser Gly Ser Leu Thr Ile Tyr	288
80 85 90	
AAC TTA ACA TCA TCA GAT GAA GAT GAG TAT GAA ATG GAA TCG CCA AAT Asn Leu Thr Ser Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser Pro Asn	336
95 100 105	
ATT ACT GAT ACC ATG AAG TTC TTT CTT TAT GTG CTT GAG TCT TCT CCA Ile Thr Asp Thr Met Lys Phe Phe Leu Tyr Val Leu Glu Ser Leu Pro	384
110 115 120 125	

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TCT CCC ACA CTA ACT TGT GCA TTG ACT AAT GGA AGC ATT GAA GTC CAA Ser Pro Thr Leu Thr Cys Ala Leu Thr Asn Gly Ser Ile Glu Val Gln 130 135 140	432
TGC ATG ATA CCA GAG CAT TAC AAC AGC CAT CGA GGA CTT ATA ATG TAC Cys Met Ile Pro Glu His Tyr Asn Ser His Arg Gly Leu Ile Met Tyr 145 150 155	480
TCA TGG GAT TGT CGT ATG GAG CAA TGT AAA CGT AAC TCA ACC AGT ATA Ser Trp Asp Cys Pro Met Glu Gln Cys Lys Arg Asn Ser Thr Ser Ile 160 165 170	528
TAT TTT AAG ATG GAA AAT GAT CTT CCA CAA AAA ATA CAG TGT ACT CTT Tyr Phe Lys Met Glu Asn Asp Leu Pro Gln Lys Ile Gln Cys Thr Leu 175 180 185	576
AGC AAT CCA TTA TTT AAT ACA ACA TCA TCA ATC ATT TTG ACA ACC TGT Ser Asn Pro Leu Phe Asn Thr Ser Ser Ile Ile Leu Thr Thr Cys 190 195 200 205	624
ATC CCA AGC AGC GGT CAT TCA AGA CAC AGA TAT GCA CTT ATA CCC ATA Ile Pro Ser Ser Gly His Ser Arg His Arg Tyr Ala Leu Ile Pro Ile 210 215 220	672
CCA TTA GCA GTA ATT ACA ACA TGT ATT GTG CTG TAT ATG AAT GGT ATT Pro Leu Ala Val Ile Thr Thr Cys Ile Val Leu Tyr Met Asn Gly Ile 225 230 235	720
CTG AAA TGT GAC AGA AAA CCA GAC AGA ACC AAC TCC AAT TGATTGGTAA Leu Lys Cys Asp Arg Lys Pro Asp Arg Thr Asn Ser Asn 240 245 250	769
CAGAAGATGA AGACAACAGC ATAACATAAT TATTTAAAAA ACTAAAAAGC CATCTGATTT CTCATTTGAG TATTACAATT TTTGAACAAC TGTTGGAAAT GTAACTTGAA GGAGCTGCTT TAAGAAGAAA TACCCACTAA CAAAGAACAA GCATTAGTT TGGCTGTCAT CAACTTATTA TATGACTAGG TGCTTGCTTT TTTGTGAGT AAATTGTTT TACTGATGAT GTAGATACTT TTGTAAATAA ATGIAAAATAT GTACACAAAGT G	829 889 949 1009 1040

## (2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 250 amino acids
  - (B) TYPE: amino acid
  - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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Met Val Ala Gly Ser Asp Ala Gly Arg Ala Leu Gly Val Leu Ser Val  
1 5 10 15

Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe Ser Gln Gln  
20 25 30

Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn  
35 40 45

Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala  
50 55 60

Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys Asn Arg  
65 70 75 80

Val Tyr Leu Asp Thr Val Ser Gly Ser Leu Thr Ile Tyr Asn Leu Thr  
85 90 95

Ser Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser Pro Asn Ile Thr Asp  
100 105 110

Thr Met Lys Phe Phe Leu Tyr Val Leu Glu Ser Leu Pro Ser Pro Thr  
115 120 125

Leu Thr Cys Ala Leu Thr Asn Gly Ser Ile Glu Val Gln Cys Met Ile  
130 135 140

Pro Glu His Tyr Asn Ser His Arg Gly Leu Ile Met Tyr Ser Trp Asp  
145 150 155 160

Cys Pro Met Glu Gln Cys Lys Arg Asn Ser Thr Ser Ile Tyr Phe Lys  
165 170 175

Met Glu Asn Asp Leu Pro Gln Lys Ile Gln Cys Thr Leu Ser Asn Pro  
180 185 190

Leu Phe Asn Thr Thr Ser Ser Ile Ile Leu Thr Thr Cys Ile Pro Ser  
195 200 205

Ser Gly His Ser Arg His Arg Tyr Ala Leu Ile Pro Ile Pro Leu Ala  
210 215 220

Val Ile Thr Thr Cys Ile Val Leu Tyr Met Asn Gly Ile Leu Lys Cys  
225 230 235 240

Asp Arg Lys Pro Asp Arg Thr Asn Ser Asn  
245 250

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 863 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: double

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## (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 18..737

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GGGGCCGCCG ACGAGCC ATG GTT GCT GGG AGC GAC GCG GGG CGG GCC CTG Met Val Ala Gly Ser Asp Ala Gly Arg Ala Leu 1 5 10	50
GGG GTC CTC AGC GTG GTC TGC CTG CTG CAC TGC TTT GGT TTC ATC AGC Gly Val Leu Ser Val Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser 15 20 25	98
TGT TTT TCC CAA CAA ATA TAT GGT GTG TAT GGG AAT GTA ACT TTC Cys Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe 30 35 40	146
CAT GTA CCA AGC AAT GTG CCT TTA AAA GAG GTC CTA TGG AAA AAA CAA His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln 45 50 55	194
AAG GAT AAA GTT GCA GAA CTG GAA AAT TCT GAA TTC AGA GCT TTC TCA Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser 60 65 70 75	242
TCT TTT AAA AAT AGG GTT TAT TTA GAC ACT GTG TCA GGT AGC CTC ACT Ser Phe Lys Asn Arg Val Tyr Leu Asp Thr Val Ser Gly Ser Leu Thr 80 85 90	290
ATC TAC AAC TTA ACA TCA TCA GAT GAA GAT GAG TAT GAA ATG GAA TCG Ile Tyr Asn Leu Thr Ser Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser 95 100 105	338
CCA AAT ATT ACT GAT ACC ATG AAG TTC TTT CTT TAT GTG CTT GAG TCT Pro Asn Ile Thr Asp Thr Met Lys Phe Phe Leu Tyr Val Leu Glu Ser 110 115 120	386
CTT CCA TCT CCC ACA CTA ACT TGT GCA TTG ACT AAT GGA AGC ATT GAA Leu Pro Ser Pro Thr Leu Thr Cys Ala Leu Thr Asn Gly Ser Ile Glu 125 130 135	434
GTC CAA TGC ATG ATA CCA GAG CAT TAC AAC AGC CAT CGA GGA CTT ATA Val Gln Cys Met Ile Pro Glu His Tyr Asn Ser His Arg Gly Leu Ile 140 145 150 155	482
ATG TAC TCA TGG GAT TGT CCT ATG GAG CAA TGT AAA CGT AAC TCA ACC Met Tyr Ser Trp Asp Cys Pro Met Glu Gln Cys Lys Arg Asn Ser Thr 160 165 170	530

- 85 -

AGT ATA TAT TTT AAG ATG GAA AAT GAT CTT CCA CAA AAA ATA CAG TGT Ser Ile Tyr Phe Lys Met Glu Asn Asp Leu Pro Gln Lys Ile Gln Cys 175 180 185	578
ACT CTT AGC AAT CCA TTA TTT AAT ACA ACA TCA TCA ATC ATT TTG ACA Thr Leu Ser Asn Pro Leu Phe Asn Thr Thr Ser Ser Ile Ile Leu Thr 190 195 200	626
ACC TGT ATC CCA AGC AGC GGT CAT TCA AGA CAC AGA TAT GCA CTT ATA Thr Cys Ile Pro Ser Ser Gly His Ser Arg His Arg Tyr Ala Leu Ile 205 210 215	674
CCC ATA CCA TTA GCA GTA ATT ACA ACA TGT ATT GTG CTG TAT ATG AAT Pro Ile Pro Leu Ala Val Ile Thr Thr Cys Ile Val Leu Tyr Met Asn 220 225 230 235	722
GGT ATG TAT GCT TTT TAAAAACAAAA TAGTTTGAAG ACTTGCATTG TTTTCCAAAG Gly Met Tyr Ala Phe 240	777
GTCAAGAAAAT ACTTTAAGGA TGAAAATAAA GTTTGAAATT TTAGACATTG GAAAAAAAAAA AAAAAAAAAAA AAAAAAAAGC GGCGCG 863	837

## (2) INFORMATION FOR SEQ ID NO:12:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 240 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Met Val Ala Gly Ser Asp Ala Gly Arg Ala Leu Gly Val Leu Ser Val 1 5 10 15
Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe Ser Gln Gln 20 25 30
Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn 35 40 45
Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala 50 55 60
Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys Asn Arg 65 70 75 80
Val Tyr Leu Asp Thr Val Ser Gly Ser Leu Thr Ile Tyr Asn Leu Thr 85 90 95

- 86 -

Ser Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser Pro Asn Ile Thr Asp  
100 105 110

Thr Met Lys Phe Phe Leu Tyr Val Leu Glu Ser Leu Pro Ser Pro Thr  
115 120 125

Leu Thr Cys Ala Leu Thr Asn Gly Ser Ile Glu Val Gln Cys Met Ile  
130 135 140

Pro Glu His Tyr Asn Ser His Arg Gly Leu Ile Met Tyr Ser Trp Asp  
145 150 155 160

Cys Pro Met Glu Gln Cys Lys Arg Asn Ser Thr Ser Ile Tyr Phe Lys  
165 170 175

Met Glu Asn Asp Leu Pro Gln Lys Ile Gln Cys Thr Leu Ser Asn Pro  
180 185 190

Leu Phe Asn Thr Thr Ser Ser Ile Ile Leu Thr Thr Cys Ile Pro Ser  
195 200 205

Ser Gly His Ser Arg His Arg Tyr Ala Leu Ile Pro Ile Pro Leu Ala  
210 215 220

Val Ile Thr Thr Cys Ile Val Leu Tyr Met Asn Gly Met Tyr Ala Phe  
225 230 235 240

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTCTTTAAAA GGCACATACA CAACACCATA

30

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

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(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AACTTATCC TTTGATTGC TTGGTACATG

30

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGCTCTGAAT TCAGATTTT TCCATAGGAC

30

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

TAAATAAACG CTATTATTT CCAGTTCTGC

30

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTAGATAGTG AGGCTTTAA AAGATGAGAA

30

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

ATACTCATCT TCATCACCTG ACACAGTGTC

30

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGTATCAGTA ATATTGATG ATGTTAAGTT

30

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AGACTCAAGC ACATATGGCG ATTCCATTTC

30

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CAATGCACAA GTTAGAACAA AGAACTTCAT

30

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CATGCATTGG ACTTCTGTGG GAGATGGAAG

30

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

TCCTCGATGG CTGTTAATGC TTCCATTAGT

30

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CATAGGACAA TCCCAGTAAT CCTCTGGTAT

30

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TATACTGGTT GAGTTTGAGT ACATTATAAG

30

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

TTGTGGAAGA TCATTACGTT TACATTGCTC

30

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TAATGGATTG CTAAGTTCCA TCTTAAAATA

30

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

TGTCAAAATG ATTGAAGTAC ACTGTATTT

30

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

TCTTGAATGA CCGCTTGATG TTGTATTAAA

30

## (2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

ATACTCATCT TCATCATACA CAACACCATA

30

## (2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CATAGGACAA TCCCATGATG ATGTTAACATT

30

## (2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 30 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TCTTGAATGA CCGCTTGAGT ACATTATAAG

30

(2) INFORMATION FOR SEQ ID NO:33:

**(i) SEQUENCE CHARACTERISTICS:**

- (A) LENGTH: 32 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser Asp Glu Asp Glu Tyr Glu  
 1                   5                   10                   15

Met Glu Ser Pro Asn Ile Thr Asp Thr Met Lys Phe Phe Leu Tyr Val  
20 25 30

(2) INFORMATION FOR SEQ ID NO:34:

**(i) SEQUENCE CHARACTERISTICS:**

(A) LENGTH: 24 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: CDS  
(B) LOCATION: 6..23

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

TCGTC GAC AAA ACT CAC ACA TGC C  
          Asp Lys Thr His Thr Cys  
           1               5

24

(2) INFORMATION FOR SEO ID NO: 35:

#### (1) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 6 amino acids

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(B) TYPE: amino acid  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

Asp Lys Thr His Thr Cys  
1 5

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

GTAAATGAGT GCGGGGGCCG CCAA

24

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 115 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

GCGGCCGCGG TCCAACCACC AATCTCAAAG CTGGTACCC GGGAAATTCAAG ATCTGCAGCA

60

TGCTCGAGCT CTAGATATCG ATTCCATGGA TCCTCACATC CCAATCCGCG CCCGC

115

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 33 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single

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(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 13..33

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GAGCCGGCCG CC ATG GTT GCT GGG AGC GAC GCG  
Met Val Ala Gly Ser Asp Ala  
1 5

33

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 7 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

Met Val Ala Gly Ser Asp Ala  
1 5

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

AAGTCGACAT AAAGAAAGAA CTTCAT

26

(2) INFORMATION FOR SEQ ID NO:41:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs

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- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

TCGACGGCGGC CGCG

14

(2) INFORMATION FOR SEQ ID NO:42:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1050 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..1041

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

ATG GTT GCT GGG AGC GAC GCG GGG CGG GCC CTG GGG GTC CTC AGC GTG	48
Met Val Ala Gly Ser Asp Ala Gly Arg Ala Leu Gly Val Leu Ser Val	
1                   5                   10                   15	

GTC TGC CTG CTG CAC TGC TTT GGT TTC ATC AGC TGT TTT TCC CAA CAA	96
Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe Ser Gln Gln	
20                   25                   30	

ATA TAT GGT GTT GTG TAT GGG AAT GTA ACT TTC CAT GTA CGA AGC AAT	144
Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn	
35                   40                   45	

GTG CCT TTA AAA GAG GTC CTA TGG AAA AAA CAA AAG GAT AAA GTT GCA	192
Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala	
50                   55                   60	

GAA CTG GAA AAT TCT GAA TTC AGA GCT TTC TCA TCT TTT AAA AAT AGG	240
Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys Asn Arg	
65                   70                   75                   80	

GTT TAT TTA GAC ACT GTG TCA GGT AGC CTC ACT ATC TAC AAC TTA ACA	288
Val Tyr Leu Asp Thr Val Ser Gly Ser Leu Thr Ile Tyr Asn Leu Thr	
85                   90                   95	

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TCA TCA GAT GAA GAT GAG TAT GAA ATG GAA TCG CCA AAT ATT ACT GAT Ser Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser Pro Asn Ile Thr Asp 100 105 110	336
ACC ATG AAG TTC TTT CTT TAT GTC GAC AAA ACT GAC ACA TGC CCA CCG Thr Met Lys Phe Phe Leu Tyr Val Asp Lys Thr His Thr Cys Pro Pro 115 120 125	384
TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC TTC CTC CCC Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro 130 135 140	432
CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC CGG ACC CCT GAG GTC ACA Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr 145 150 155 160	480
TGC GTG GTG GTG GAC GTG AGC CAC GAA GAC CCT GAG GTC AAG TTC AAC Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn 165 170 175	528
TGG TAC GTG GAC GGC GTG GAG GTG CAT AAT GCC AAG ACA AAG CGG CGG Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg 180 185 190	576
GAG GAG CAG TAC AAC AGC ACC TAC CGG GTG GTC AGC GTC CTC ACC GTC Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val 195 200 205	624
CTG CAC CAG GAC TGG CTG AAT GGC AAG GAG TAC AAG TGC AAG GTC TCC Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser 210 215 220	672
AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA GCC AAA Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys 225 230 235 240	720
GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC CCA TCC CGG GAT Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp 245 250 255	768
GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC TGC CTG GTC AAA GGC TTC Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe 260 265 270	816
TAT CCC AGC GAC ATC GCC GTG GAG TGG GAG AGC AAT GGG CAG CCG GAG Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu 275 280 285	864
AAC AAC TAC AAG ACC ACG CCT CCC GTG CTG GAC TCC GAC GGC TCC TTC Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe 290 295 300	912
TTC CTC TAC AGC AAG CTC ACC GTG GAC AAG AGC AGG TGG CAG CAG CGG Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly 305 310 315 320	960

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AAC GTC TTC TCA TGC TCC GTG ATG CAT GAG GCT CTG CAC AAC CAC TAC Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr 325	330	335	1008
ACG CAG AAG AGC CTC TCC CTG TCT CCG GGT AAA TGACTGCCGG Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys 340		345	1050

## (2) INFORMATION FOR SEQ ID NO:43:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 347 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Met Val Ala Gly Ser Asp Ala Gly Arg Ala Leu Gly Val Leu Ser Val 1 5 10 15
Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe Ser Gln Gln 20 25 30
Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn 35 40 45
Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala 50 55 60
Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys Asn Arg 65 70 75 80
Val Tyr Leu Asp Thr Val Ser Gly Ser Leu Thr Ile Tyr Asn Leu Thr 85 90 95
Ser Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser Pro Asn Ile Thr Asp 100 105 110
Thr Met Lys Phe Phe Leu Tyr Val Asp Lys Thr His Thr Cys Pro Pro 115 120 125
Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro 130 135 140
Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr 145 150 155 160
Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn 165 170 175
Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg 180 185 190

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Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val  
195 200 205

Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser  
210 215 220

Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys  
225 230 235 240

Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp  
245 250 255

Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe  
260 265 270

Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gln Pro Glu  
275 280 285

Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe  
290 295 300

Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly  
305 310 315 320

Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr  
325 330 335

Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys  
340 345

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CLAIMS

We claim:

1. A polypeptide having the amino acid sequence:  $X_1-X_2$ -(SEQ ID NO:1) Asn Arg Val Tyr Leu Asp Thr Val Ser Gly-Y, wherein:

$X_1$  is hydrogen or methionyl;

$X_2$ , if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy-terminal 1 to 77 amino acids of the sequence (SEQ ID NO:5): Val Ala Gly Ser Asp Ala Gly Arg Ala Leu Gly Val Leu Ser Val Val Cys Leu Leu His Cys Phe Gly Phe Ile Ser Cys Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser Phe Lys;

Y is hydroxyl or a polypeptide of the following amino acid sequence or a portion thereof consisting of the amino terminal 1 to 32 amino acids of the sequence (SEQ ID NO:33): Ser Leu Thr Ile Tyr Asn Leu Thr Ser Ser Asp Glu Asp Glu Tyr Glu Met Glu Ser Pro Asn Ile Thr Asp Thr Met Lys Phe Phe Leu Tyr Val;

and analogs and derivatives thereof,  
said polypeptide being capable of binding to CD2.

2. The polypeptide according to claim 1  
wherein,

$X_2$ , if present, is a polypeptide having the following amino acid sequence or a portion thereof consisting of the carboxy terminal 1 to 50 amino acids of the sequence (SEQ ID NO:2): Phe Ser Gln Gln Ile Tyr Gly Val Val Tyr Gly Asn Val Thr Phe His Val Pro Ser Asn Val Pro Leu Lys Glu Val Leu Trp Lys Lys Gln Lys Asp Lys

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Val Ala Glu Leu Glu Asn Ser Glu Phe Arg Ala Phe Ser Ser  
Phe Lys;

Y is hydroxyl or a polypeptide of the following  
amino acid sequence or a portion thereof consisting of  
the amino-terminal 1 to 10 amino acids of the sequence  
(SEQ ID NO:3): Ser Leu Thr Ile Tyr Asn Leu Thr Ser  
Ser;

and analogs and derivatives thereof,  
said polypeptide being capable of binding to CD2.

3. The polypeptide according to claim 1,  
selected from the group consisting of polypeptides  
having the amino acid sequences: amino acids 29-120 of  
SEQ ID NO:10, amino acids 29-108 of SEQ ID NO:10, amino  
acids 48-108 of SEQ ID NO:10, and SEQ ID NO:7.

4. The polypeptide according to claim 2  
having the amino acid sequence (SEQ ID NO:7): Lys Asn  
Arg Val Tyr Leu Asp Thr Val Ser Gly Ser Leu Thr Ile  
Tyr.

5. An isolated DNA sequence encoding a  
polypeptide of any of claims 1, 2, 3 or 4.

6. An isolated DNA sequence encoding the  
CD2-binding domain of LFA-3 comprising:

(5') (SEQ ID NO:6) AATAGGGTTT ATTTAGACAC  
TGTGTCAGGT (3').

7. An isolated DNA sequence comprising a  
DNA sequence encoding the CD2-binding domain of LFA-3  
selected from the group consisting of the DNA inserts  
of pPYM57, pPMDRM54-6, pPMDRM55-9, pPMDRM56-C,  
pPMDRM58-15, pPYM63-4, pPYM65-8, pPMDRM100-4,  
pPMDRM101-1, pPMDRM102-8, and pPMDRM3-10.

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8. A recombinant DNA molecule comprising a DNA sequence according to claim 5, 6 or 7 and an expression control sequence, wherein the expression control sequence is operatively linked to the DNA sequence.

9. A fusion protein, having an amino terminal region characterized by the amino acid sequence of the polypeptides of any one of claims 1-4 and having a carboxy terminal region comprising a domain of a protein or polypeptide other than LFA-3.

10. The fusion protein according to claim 9 wherein the carboxy terminal region comprises at least a portion of the Fc region of an immunoglobulin.

11. The fusion protein according to claim 10 wherein the portion of the Fc region of an immunoglobulin comprises a hinge region and C<sub>H</sub>2 and C<sub>H</sub>3 constant domains.

12. The fusion protein according to claim 10 wherein the portion of the Fc region comprises a portion of a hinge region that is capable of forming intermolecular disulfide bonds and C<sub>H</sub>2 and C<sub>H</sub>3 constant domains.

13. The fusion protein according to claim 12 which is amino acids 29-347 of SEQ ID NO:43.

14. An isolated DNA sequence coding for a fusion protein according to any one of claims 9-13.

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15. A recombinant DNA molecule comprising the DNA sequence according to claim 14 and an expression control sequence, wherein the expression control sequence is operatively linked to the DNA sequence.

16. The recombinant DNA molecule, according to claim 15, selected from the group consisting of plasmid pSAB152 and plasmid pMDR(92)Ig-3.

17. A multimeric protein capable of binding to CD2 comprising (a) two or more polypeptides according to any one of claims 1-4, (b) two or more fusion proteins according to any one of claims 9-13, or (c) one or more polypeptides according to any one of claims 1-4 and one or more fusion proteins according to any one of claims 9-13.

18. A method for producing a protein comprising the step of culturing a host cell transformed with the recombinant DNA molecule of claim 8, 15 or 16.

19. A host cell transformed with the recombinant DNA molecule according to claim 8, 15 or 16.

20. A method for labeling CD2<sup>+</sup> cells or proteins containing the LFA-3 binding domain of CD2, said method comprising the step of incubating the CD2<sup>+</sup> cells or proteins containing the LFA-3 binding domain of CD2 with a polypeptide according to any one of claims 1-4 or a fusion protein according to any one of claims 9-13.

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21. A method for clearing solutions, including sera, of anti-LFA-3 antibodies recognizing LFA-3 epitopes other than the CD2-binding domain of LFA-3 comprising the steps of contacting the solution of anti-LFA-3 antibodies with an LFA-3 deletion mutant protein selected from M57, M55, M56, PIM3, M100, or combinations thereof, under conditions suitable for forming antibody-protein complexes, then removing any antibody-protein complexes formed from said solution.

22. A method for isolating CD2<sup>+</sup> cells or proteins having the LFA-3-binding domain of CD2 comprising the steps of coupling a polypeptide according to any one of claims 1-4 or a fusion protein according to any one of claims 9-13 to a chromatographic substrate and contacting a cell suspension containing CD2<sup>+</sup> cells or a solution containing proteins having the LFA-3-binding domain of CD2 with said substrate under conditions suitable for the formation of a LFA-3/CD2 complex.

23. A pharmaceutical composition comprising a polypeptide according to any one of claims 1-4 or a fusion protein according to any one of claims 9-13, and a pharmaceutically acceptable carrier.

24. A diagnostic kit for detecting the presence of CD2<sup>+</sup> cells or LFA-3-binding proteins comprising a polypeptide according to any one of claims 1-4 or a fusion protein according to any one of claims 9-13, as a reagent.

25. The use of a polypeptide according to any one of claims 1-4 or a fusion protein according to any one of claims 9-13 to inhibit T-cell activation.

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26. The use of a polypeptide according to any one of claims 1-4 or a fusion protein according to any one of claims 16-19 to inhibit proliferation of peripheral blood lymphocytes.

27. A method for initiating T-lymphocyte responses in vivo comprising administering to a mammal a polypeptide according to any one of claims 1-4 or a fusion protein according to any one of claims 9-13.

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**FIG. 1A-1**

1	CGACGGGCCATGGTGTGCTGGGAGCGGGGGCCCTGGGGTCC MetValAlaGlySerAspAlaGlyArgAlaLeuGlyValle -28	50
51	CAGCGTGGTCTGCCTGCACTGCTTTCAGCTGTTTCCC uSerValValCysLeuLeuHisCysPheGlyPheIleSerCysPheSerG +1	100      3
101	AACAAATATGGTGTGTTGGAATGTAATGTAATTCCATGTACCAAGC InGlnIleTerGlyValValTygGlyAsnValThrPheHisValProSer M53	150      19
151	AATGTGCCCTTAAAGAGGTCCATGGAAACAAAGGATAAGTGC AsnValProLeuValGluValValTygGlyAsnValThrPheHisValProSer M54	200      M55
201	AGAACTGGAAAATTCTGAATTCAAGAGCTTCTCATCTTTAAATAAGGG aGluLeuGluAsnSerGluPheArgAlaPheSerSerPheLysAsnArgV M56	250      53
251	TTTATTAGACACACTGTCAGGTAGCCTCACTATCTACAACATCA alTyrLeuAspThrValSerGlySerLeuThrIleTyrAsnLeuThrSer M57	300      69

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301	TCAGATGAAAGATGAGTATGAAATCGCCAATATTACTGATA <u>CCAT</u>	350
	<u>Se</u> <u>Asp</u> <u>Glu</u> <u>Asp</u> <u>Glu</u> <u>Ty</u> <u>r</u> <u>Glu</u> <u>Met</u> <u>Glu</u> <u>Ser</u> <u>Pro</u> <u>Asn</u> <u>Ile</u> <u>Thr</u> <u>Asp</u> <u>Thr</u> <u>Me</u>	86
	M59	M60
351	GAA <del>G</del> TCTTCTTTATGTGCTTGAGTCTCTCCATCTCCCACACTAACTT <u>Lys</u> <u>Phe</u> <u>Phe</u> <u>Leu</u> <u>Ty</u> <u>r</u> <u>Va</u> <u>Ile</u> <u>Leu</u> <u>Glu</u> <u>User</u> <u>Pro</u> <u>Ser</u> <u>Pro</u> <u>Thr</u> <u>Leu</u> <u>Thr</u> <u>C</u>	400
	M61	M62
401	GTGCATTTGACTAATGGAAGCATTGAAAGTCCAAATGCATGATA <u>CCAGAGCAT</u>	450
	<u>Y</u> <u>s</u> <u>A</u> <u>l</u> <u>a</u> <u>Leu</u> <u>Thr</u> <u>Asn</u> <u>Gly</u> <u>Ser</u> <u>Ile</u> <u>Glu</u> <u>Va</u> <u>l</u> <u>Gln</u> <u>Cys</u> <u>Met</u> <u>Ile</u> <u>Pro</u> <u>Glu</u> <u>His</u>	119
	M63	M64
451	TACAACAGCCATCGAGGACTTATAATGTACTCATGGGATTGTCCTATGGA <u>Tyr</u> <u>Asn</u> <u>Ser</u> <u>His</u> <u>Arg</u> <u>Gly</u> <u>Leu</u> <u>Ile</u> <u>Met</u> <u>Tyr</u> <u>Ser</u> <u>Trp</u> <u>Asp</u> <u>Cys</u> <u>Pro</u> <u>Met</u> <u>G1</u>	500
	M65	M66
501	GCAATGTAACGTAACCTCAACCATAATAATTAAAGATGGAAAATGATC <u>u</u> <u>Gln</u> <u>Cys</u> <u>Lys</u> <u>Arg</u> <u>Asn</u> <u>Ser</u> <u>Thr</u> <u>Ser</u> <u>Ile</u> <u>Leu</u> <u>Pro</u> <u>Phenyl</u> <u>Lys</u> <u>Met</u> <u>Glu</u> <u>Asn</u> <u>Asp</u> <u>L</u>	550

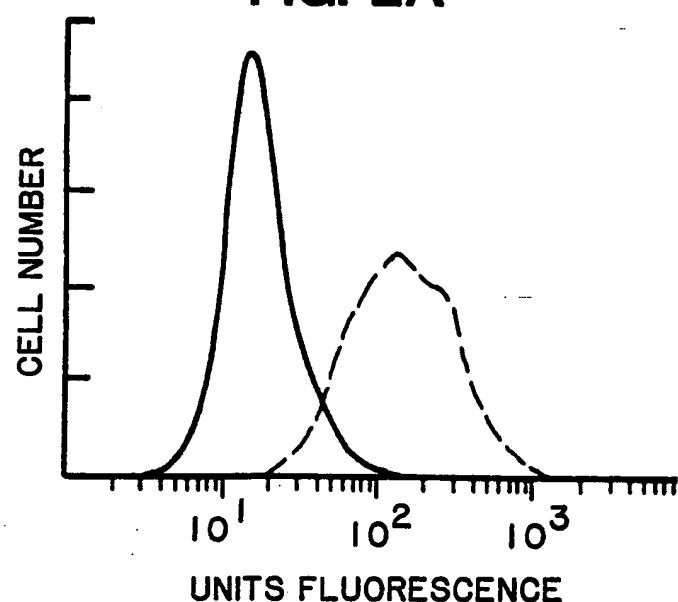
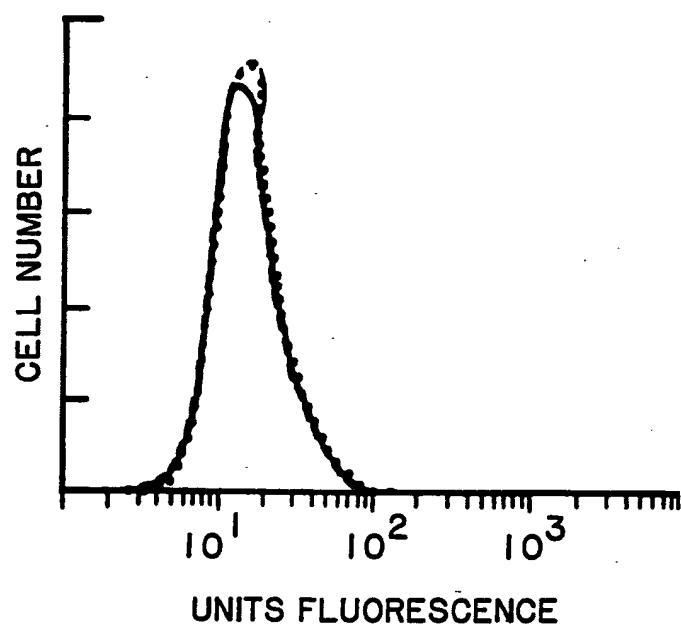
**FIG. 1A-2**

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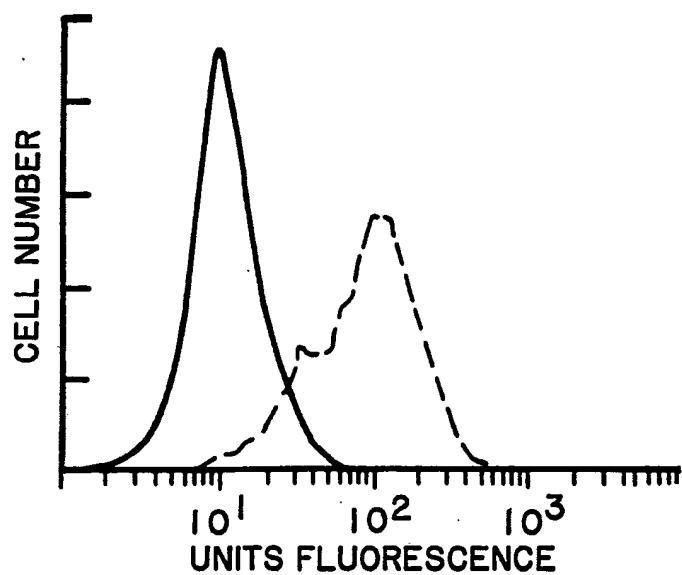
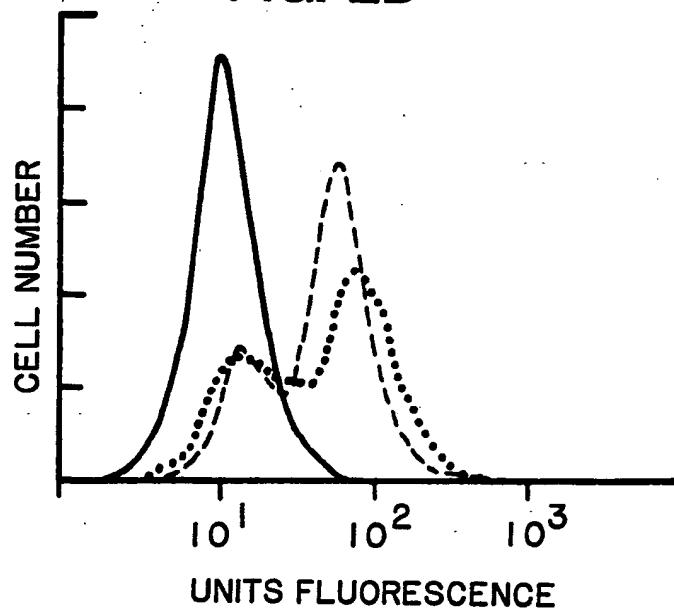
18

551	TTC CAC AAA AATA CAG GT TACT CT TAG CAAT CC ATT ATT TA AT CA ACA eu Pro Gln Lys Ile Glu Cys Thr Leu Ser Asn Pro Leu Phe Asn Thr Thr	600 169
601	TCAT CAAT CAT TTG AC CAAC CT GT AT CC CAAG CG CG TC ATT CA AG AC A Ser Ser Ile Leu Thr Thr Cys Ile Pro Ser Gly His Ser Arg Ile	650 186
651	CAG AT AT GC ACT TAT ACC CATT AGC CAG TA ATT CAC AAC AT GT ATT G SArg Ty Ile Al a Leu Ile Pro Ile Pro Leu Val Ile Leu Thr Cys Ile Val Transmembrane	700 203
701	TG CT GT AT AT GG T ATT CT G AA AT GT G AC AG AA ACC CAG AC AG A ACC al Leu Tyr Met Arg Gly Ile Leu Ile Cys Asp Arg Gly Pro Asp Arg Thr	750 219
751	AA CT CC AA ATT GT ATT GG T AA AC AGA AG AT GA AG AC AA CAG CC ATA A CT AA ATT Asn Ser Asn	800 222
801	AT TT T AAA AAC T AAA AG CC AT CT GT GAT T CT CAT TT GT GAG T ATT CAA ATT .	850
851	TT GAA CAA ACT GT T GG AA AT GT AA CT GT GAG CT GCT TT TA AG A AG A AT .	900
901	AC CC ACT A ACA A AGA ACA AGC ATT AG T TT GG CT GT C AT CA ACT T ATT AT .	950
951	AT G ACT A TAG GT GCT TT GCT TTT TGT CAG TA ATT TG TTT TACT GT AT G AT G .	1000
1001	TAG AT ACT TT GT TAA ATA AT GT TAA ATA AT GT AC ACA AGT G .	1040

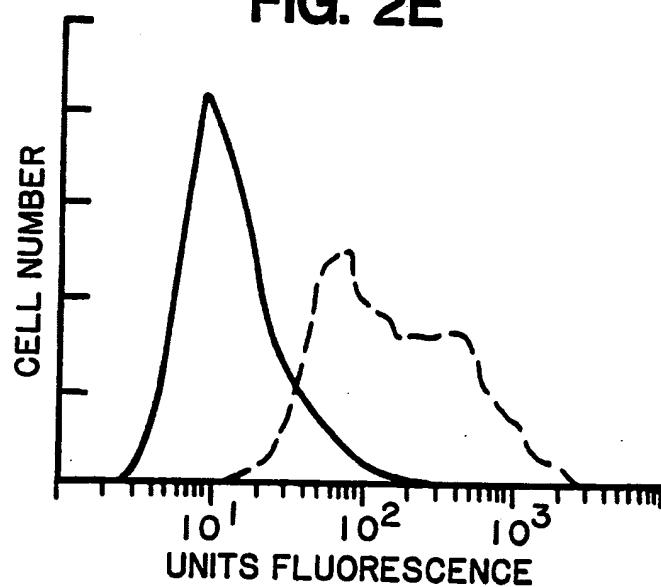
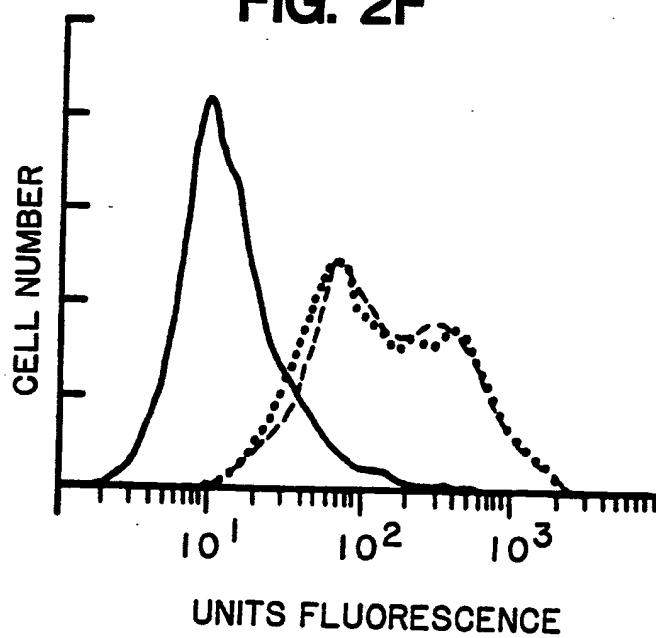
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**FIG. 2A****FIG. 2B****SUBSTITUTE SHEET**

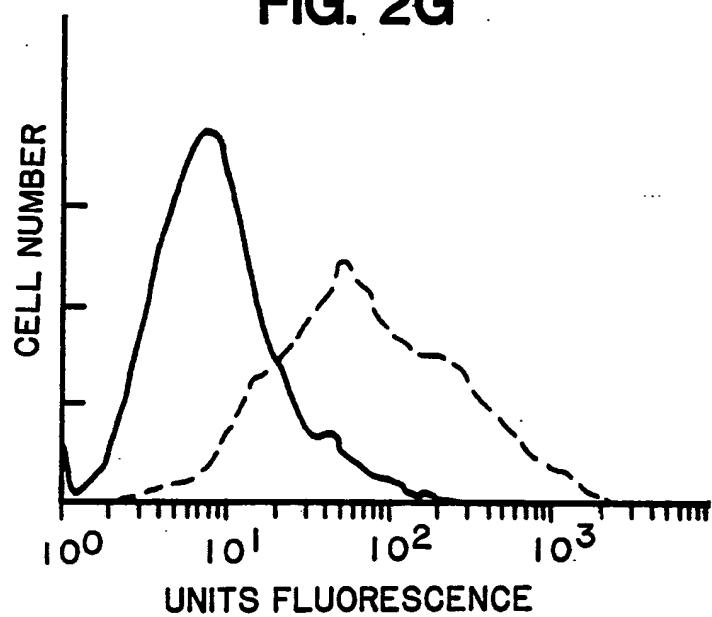
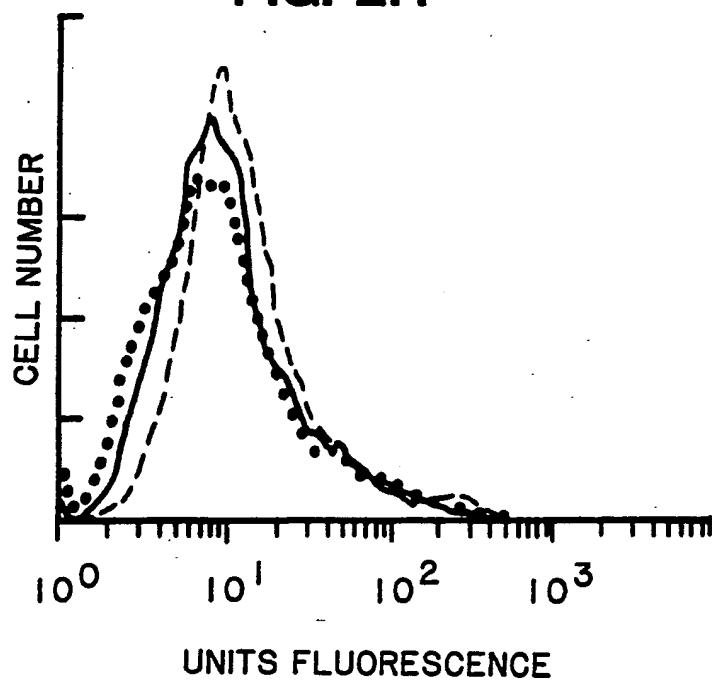
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**FIG. 2C****FIG. 2D****SUBSTITUTE SHEET**

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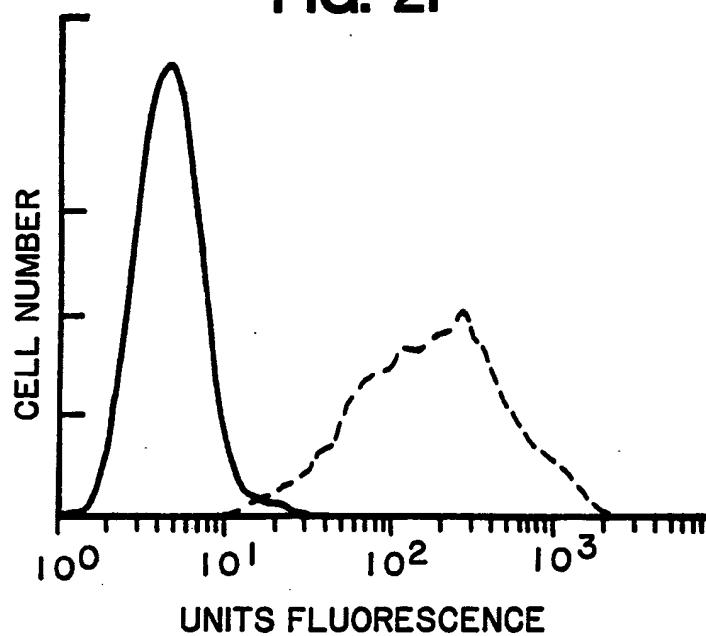
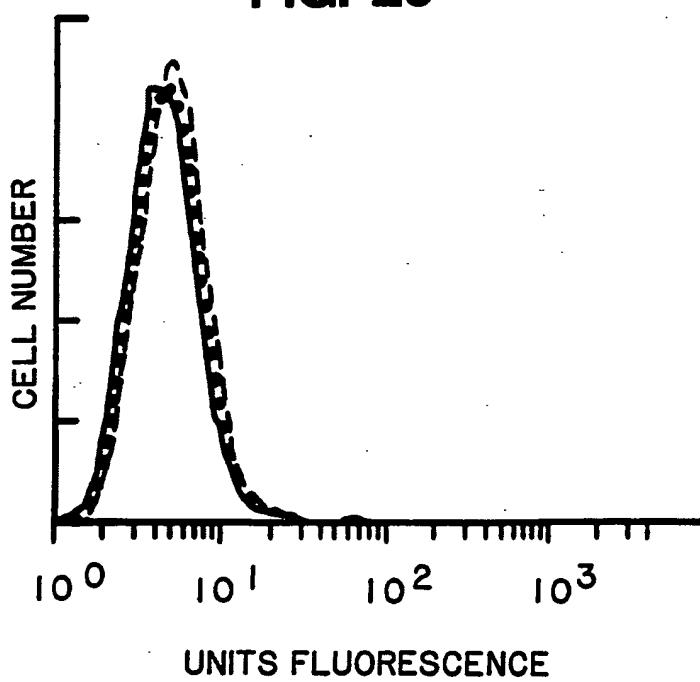
**FIG. 2E****FIG. 2F****SUBSTITUTE SHEET**

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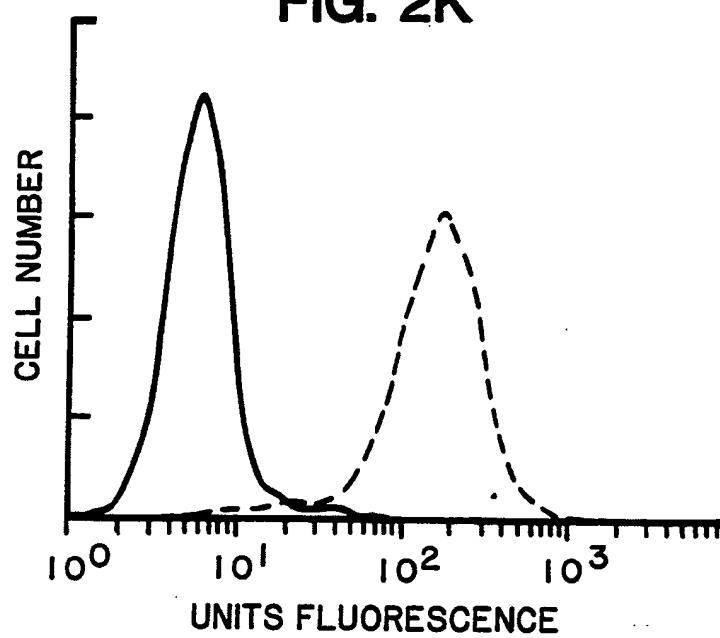
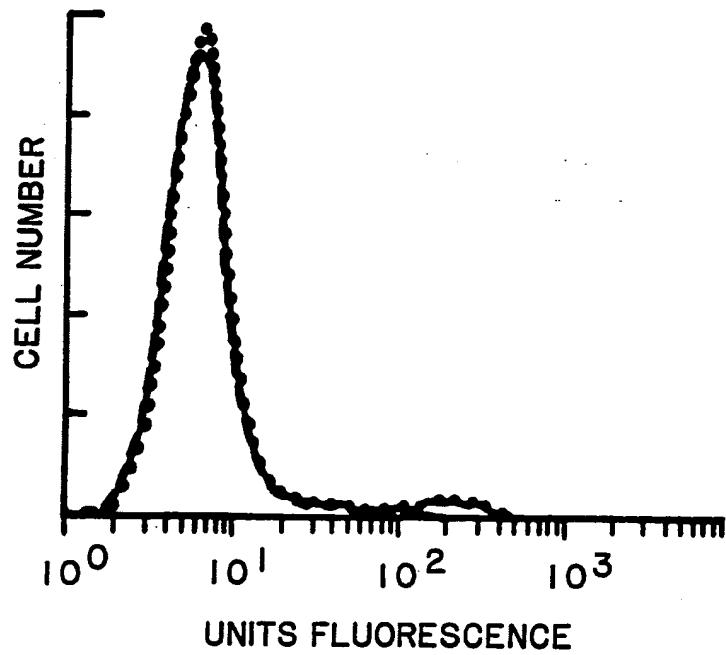
**FIG. 2G****FIG. 2H**

SUBSTITUTE SHEET

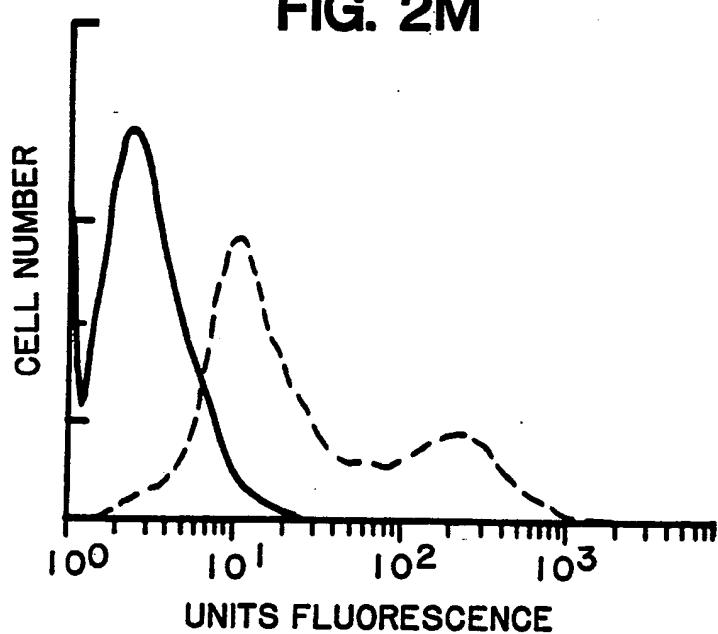
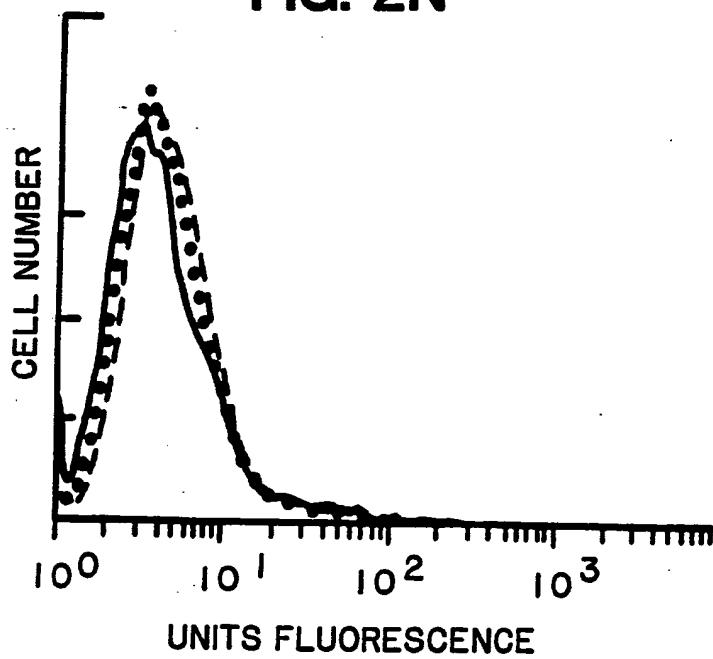
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**FIG. 2I****FIG. 2J****SUBSTITUTE SHEET**

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**FIG. 2K****FIG. 2L****SUBSTITUTE SHEET**

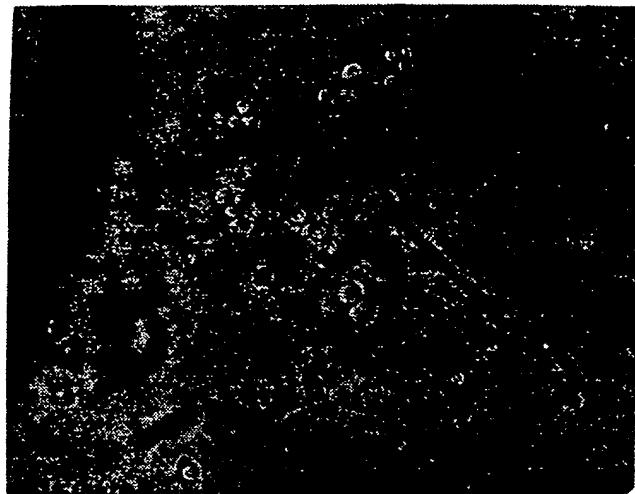
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**FIG. 2M****FIG. 2N**

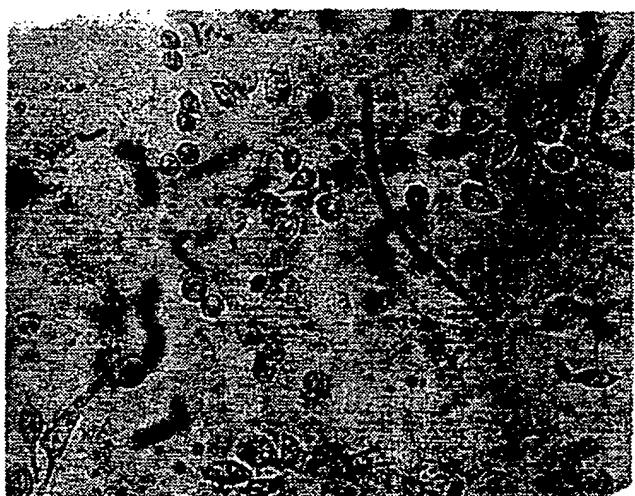
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**FIG. 3A**



**FIG. 3B**



**FIG. 3C**



**SUBSTITUTE SHEET**

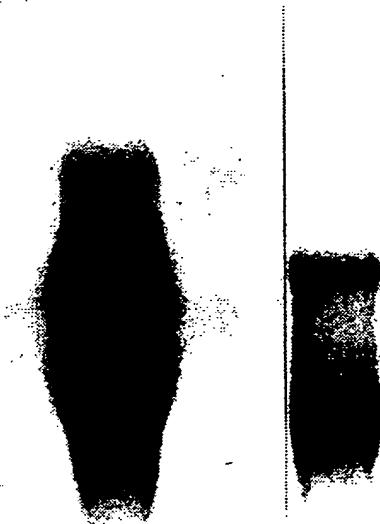
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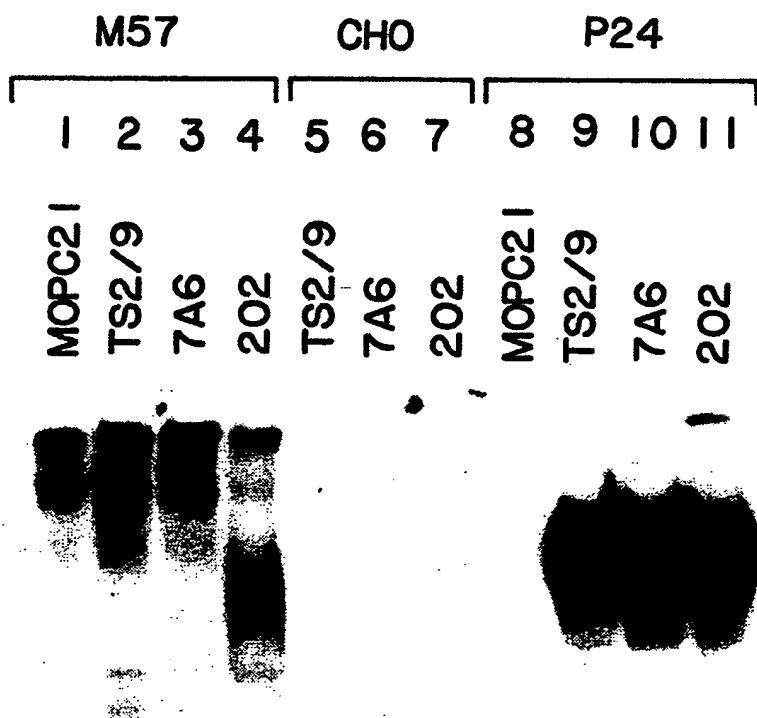
**FIG. 4**

**CHO M57      M16,3**



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**FIG. 5****SUBSTITUTE SHEET**

**FIG. 6A-1**

1 CGACGAGCCATGGTTGCTGGAGCCGACGGGGCCCTGGGGCCT  
MetValAlaGlySerAspAlaGlyArgAlaLeuGlyValle  
-28

51 CAGCGTGGTCTGCCCTGCTGCACCTGCTTGTTTCATCGCTGTGTTCCC  
uSerValValCysLeuHisCysPheGlyPheLeuSerCysPheSerG  
50  
3  
+1

101 ACAAAATATGGTGTGTTGTAATGGAAATGTAACCTCCATGTACCAAGC  
InGlnIleTygGlyValValTygGlyAsnValThrPheHisValProSer  
19

151 AATGTGCCTTAAAGAGGTCCATTGGAAAAAACAAAAGGATAAAAGTTGC  
AsnValProLeuLeuGluLeuTriPlysLeuGlnLysAspLysValAl  
36

201 AGAACTGGAAATTCTGAATTCAAGGTTCTCATCTTTAAATAAGGG  
aGluLeuGluAsnSerGluPheLeuSerSerPheLeuSerLeuArgV  
250  
53

M 100

251 TTTATTAGACACTGTGTCAGGTAGCCTCACTATCTACAACCTAACATCA  
alTyrLeuAspThrValSerGlySerLeuThrIleLeuThrSer  
300  
69

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301	TCAGATGAAGATGAGTATGAAATTGGAATCGCCAAATTACTGATAACC SerAspGluAspGluTyrglumetGluserProAsnIleThrAspThrMe	350 86
351	GAAGTTCTTCTTTATGGCTTGAGTCTTCCATCTCCACACTAACT tLysPhePheLeuTyrvAlleuGluSerIleuProSerProThrLeuThrC M 101	400 103
401	GTGCATGACTTAATGGAAGCATGAACTCCAATGCATGATAACCAGAGCAT ysAlaLeuThrAsnGlySerIleGlyValGlnCysMetIleProGluHis	450 119
451	TACAACAGCCATCGAGGACTTATAATGTACTCATGGATTGCCCTATGGA TyrAsnSerHisArgGlyLeuIleMetTyrSerIlePheAspCysProMetG1	500 136
501	GCAATGTAACCGTAACCTAACCGTATATTTTAAGATGGAAATGATC uGlnCysLysArgAsnSerThrSerIleIlePheIleAspMetGluAsnAspL M 102	550 153

**FIG. 6A-2**

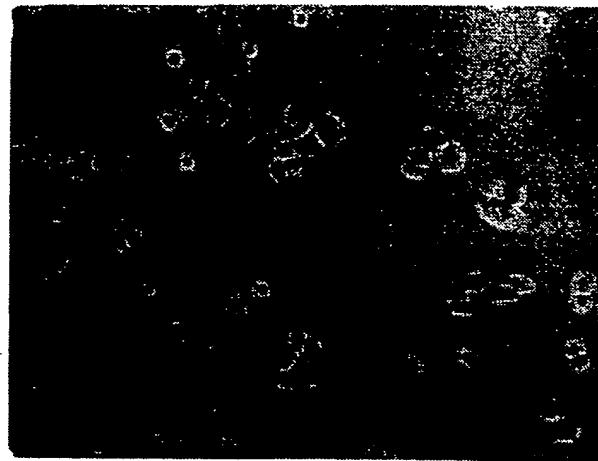
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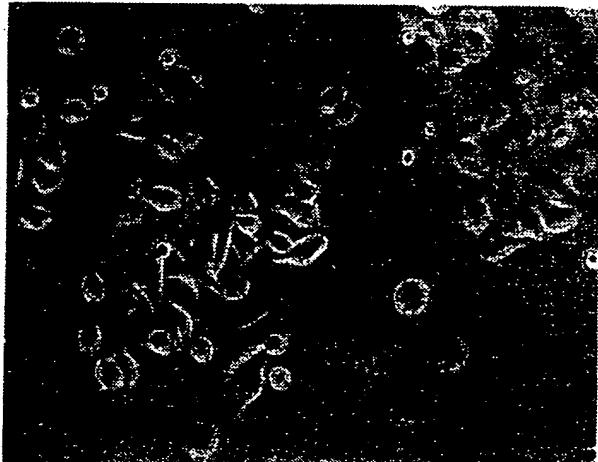
**FIG. 6B**

551	TTCCACAAAAATACAGTGTACTCTTAGCAAATCCATTATTAAATAACAACA euProGlnLysIleGlnCysThrLeuSerAsnProLeuPheAsnThrThr	600 169
601	TCATCAATTGACAAACCTGTATCCCAGCAGGGTCATTCAAGACAA <u>SerSerIleIleLeuThrThrCysIleProSerSerGlyHisSerArgHi</u>	650 186
651	CAGATATGCCACTTATACCCATAACCATTAGCAGTAATTACAACATGTATTG SArgTYRAlaLeuIleProLeuAlaValIleIleThrThrCysIle	700 203
701	TGCTGTATATGAATGGTATTCTGAAATGTGACAGAAAACCAGACAGAACCC alLeuTyrmMetAsnGlyIleLeuLysCysAspArgLysProAspArgThr	750 219
751	AACTCCAATTGATTGGTAACAGAAGATGAAGACAAACAGCATAACTAAATT AsnSerAsn	800 222
801	ATTTTAAAAACTAAAAGCCATCTGATTCTCATTTGAGTATTACAATT TTGAAACAACGTGTTGAAATGTAACCTGAAAGCAGCTGCTTTAAGAAGAAAT	850
851	ACCCACTAACAAAGAACAAAGCATTAGTTGGCTGTCACTTATTAT ATGACTAGGTGCTTGCTTTTGTCAAGTAATTGTTTACTGTGATG	900
901	ATGACTAGGTGCTTGCTTTTGTCAAGTAATTGTTTACTGTGATG	950
951	TAGATACTTTGTTAAATAATGTAATAATGTAACACAAAGTG	1000
1001		1040

**FIG. 7A**

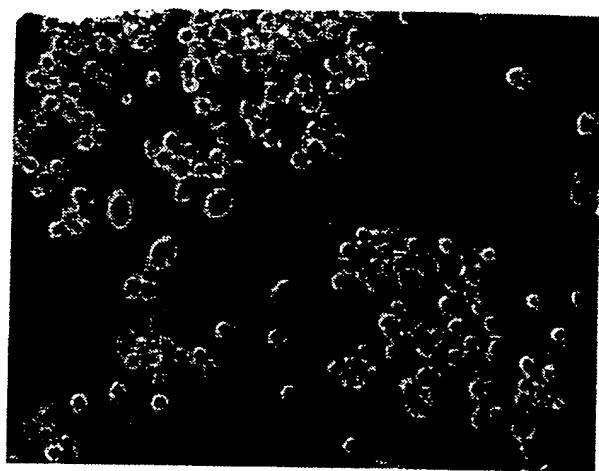


**FIG. 7B**

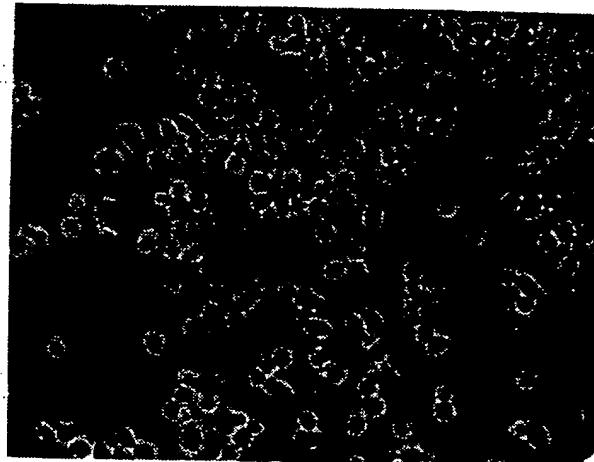


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**FIG. 7C**

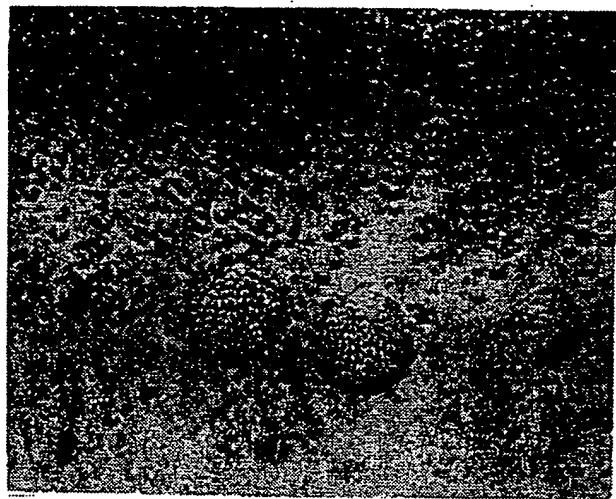


**FIG. 7D**



**SUBSTITUTE SHEET**

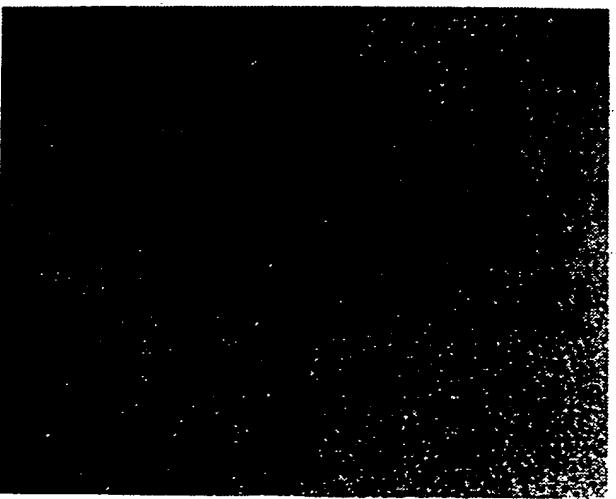
**FIG. 8A**



**FIG. 8B**



**FIG. 8C**



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**FIG. 9A**

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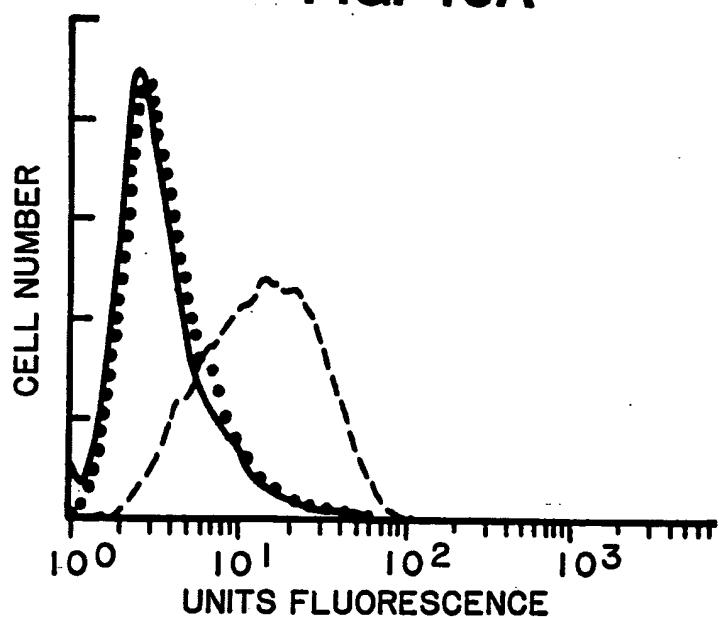
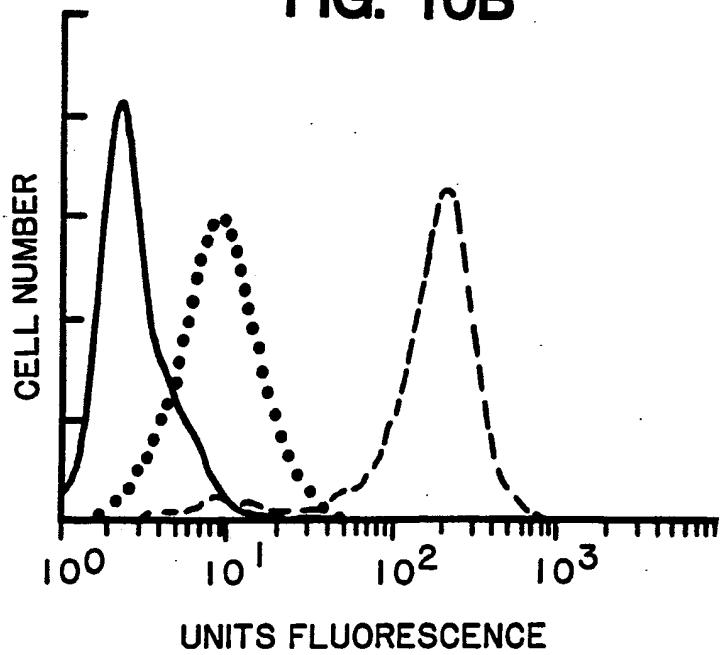
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401	ACTAACTTGTGCATTGACTTAATGGAAGCCATTGAAGTCCAATGCCATGATA Ile <u>ThrCysAla</u> Leu <u>ThrAsnGlySerIleGluValGlnCysMet</u> TleP	450
451	CAGAGCATTACAAACAGCCATCGAGGACTTATAATGTTACTCATGGGATTGT Ile <u>GluHistYrasnSerHisArgGlyLeuIleMetTyrsertDAspCys</u>	500
501	CCTATGGAGCAATGTAAACGTAACCTAACCAACAGTATATTTAAGATGGA <u>PrometGluGlnCysLysArgAsnSerthrSerIleTerPheLysMetG1</u>	550
551	AAATGATCTTCCACAAAAATAACAGTGACTCTTAGCAATCCATTATTAA <u>AsnAspLeuProGlnLysIleGlnCysThrLeuSerAsnProLeuPheA</u>	+150
601	ATACAACATCATCAATTCAATTGACAACCTGTATCCCCAAGCAGGGTCAT SerThrSerSerIleLeuThrThrCysIleProSerSerGlyHis	650
651	TCAAGACACAGATAATGCACCTATAACCCATACCATTAGCAGTAATTACAAC SerArgHisArgTyralaileProIleProLeuAlaValIleThrTh	700
701	ATGTATTGTGCTGTATGAATGGTATGTTGCTTTAAACAAATAG rcysIleValLeuTyrmetaAsnGlyMetTyraPhe	+200
751	TTTGAAGAAACTTGCAATTGTTCCAAAGGTCAAGAAATAGTTAAGGATGA AAATAAAGTTGAAATTAGACATTGAA	750
801	AAAAGGGCCGC	800
851		850
		863

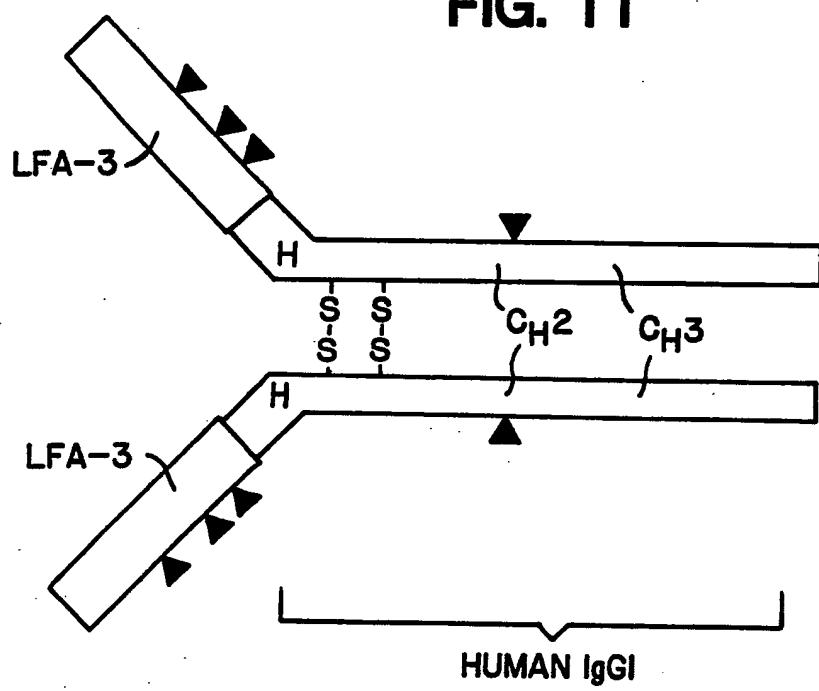
**FIG. 9B**

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**FIG. 10A****FIG. 10B****SUBSTITUTE SHEET**

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**FIG. 11****SUBSTITUTE SHEET**

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**FIG. 12A**

**LFA-3**

ATGGTTCGAGCGAACGGGGCCCTGGGTCTACGGTGTT	50
MetvalAlaGlySerAspAlaGlyLeuGlyValleuSerValva	
CTGCCTGCTGCACTGCTTGGTTCATCAGCTGTTTCCCAAATAAT	51
IcysLeuLeuHisCysPheGlyPheIleSerCysPheSerGlnGlnIleF	
ATGGTGTGTTGTTGGAAATGTAACCTTCCATGTACCAAGCAAATGTTGCT	101
yrGlyValValtyrGlyAsnValThrPheHisValProSerAsnValPro	
TAAAAGAGGTCTATGGAAAAAGGATAAAAGTTGCAGAACTGGA	151
LeuLysGluValLeutriPlysLysGlnLysAspLysValAlaGluLeuG1	
AAATTCTGAATTAGAGCTTCTCATCTTTAAATAAGGGTTATTAG	201
wasnSerGluPheArgAlaPheSerSerPhelysAsnArgValtyrLeuA	
AAACTGTTGCTTGGCTCACTATCTACAACCTAACATCATCAGATGAA	251
sphThrValSerGlySerLeuThrIleTyrasnLeuThrSerAspGlu	
GATGAGGTATGAAATGGAAATGCCAAATATTACTGATAACCATGAAGTTCTT	301
AspGluIutyrglumetGluSerProAsnIleThrAspThrMetLysPhePh	
LFA-3 → Hinge.	351
TCTTATGTOGACAAACTCACACATGCCAACCTGAAAC	
eleutyrvalAspLysThrHisThrCysProProCysPheIleProGluL	
TCCCTGGGGGACCCGTCACTCTCTCCCCAAACCCAAAGGACACC	401
euLeuGlyGlyProSerValPheLeuPheProProLysProLysAspThr	
CTCATGATCTCCGGACCCCTGAGGTCACTGCGTGGTGGACGTCAG	451
LeuMetIleSerArgThrProGluValThrCysValValAspValSe	
CCACGGAGACCCCTGAGGTCAAGTTCAACTGGTACGTTGGACGGCGTGGAGG	501
rhisGluAspProGluValLysPheAsnTriptyrValAspGlyValGluV	

CH2

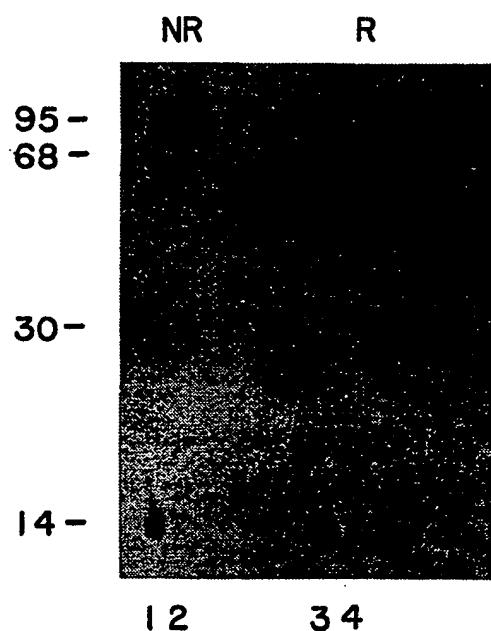
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**FIG. 12B**

551 TGCATAATGCCAAGACAAAGCCGGGAGCCAGTACAACAGCACGTAC  
 alHisAsnAlaLysThrLysProArgGluGluGlnLysAsnSerThrTrp 600  
 601 CGGGTGGTCAGCGTCCCTCACCGTCCCTGCAACCAGGACTGGCTGAATGGCAA 650  
 ArgValValSerValLeuThrValLeuHisGlnAspTripleuAsnGlyLys  
 651 GGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCAGCCCCATCGAGA  
 sGluTyrlsCysBlysValSerAsnLysAlaLeuProAlaProIleGluL  
 CH2 → CH3  
 701 AAACCATCTCCAAGCDAAGGGCAGCCCCGGAGAACCAACAGGTGTACACC  
 ysThrIleSerLysAlaLysGlyGlnProArgGluProGlnValThrThr 750  
 751 CTGGCCCCATCCGGGATGAGCTGACCAAGAACCAAGGTCAAGCCTGACCTG  
 LeuProProSerArgAspGluLeuThrLysAsnGlnValSerLeuThrCY  
 801 CCTGGTCAAAGGCTTCATCCAGCGACATCGCCGTTGGAGTGGAGAGCCA 800  
 sLeuValLysGlyPheTyrlProSerAspIleAlaValGluLysGluSerA  
 851 ATGGGAGGCCGGAGAACAAACTACAAGACCAAGCTCCCGTGTGGACTCC  
 snglyGlnProGluAsnAsnTyrlsThrProValLeuAspSer 850  
 901 GACGGCTCCCTTCCTCTACAGCAAGCTCACCGTGGACAAGAGGGCTCTGGACA  
 AspGlySerPhePheLeutyrSerLysLeuThrValAspLysSerArgTrp 950  
 951 GCAGCAGGGAAACGTCTCATGCTCCGTGATGCATGAGGTCTGGACA  
 pGlnGlyAsnValPheSerCysSerValMetHisGluAlaLeuHisA 1000  
 1001 ACCACTACACGCCAGAACGACCTCTCCCTGTCCTCCGGTAAATGAGTGGCG  
 snHistYrThrGlnLysSerLeuSerLeuSerProGlyLys 1050

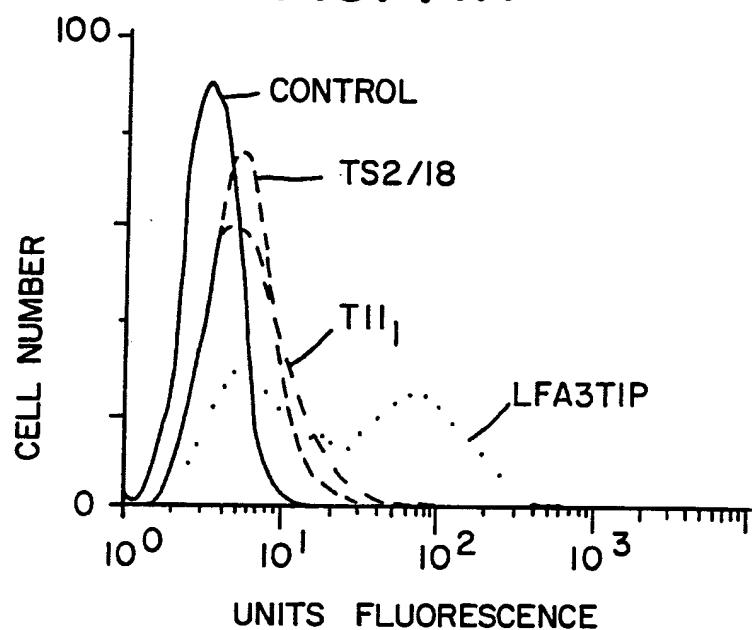
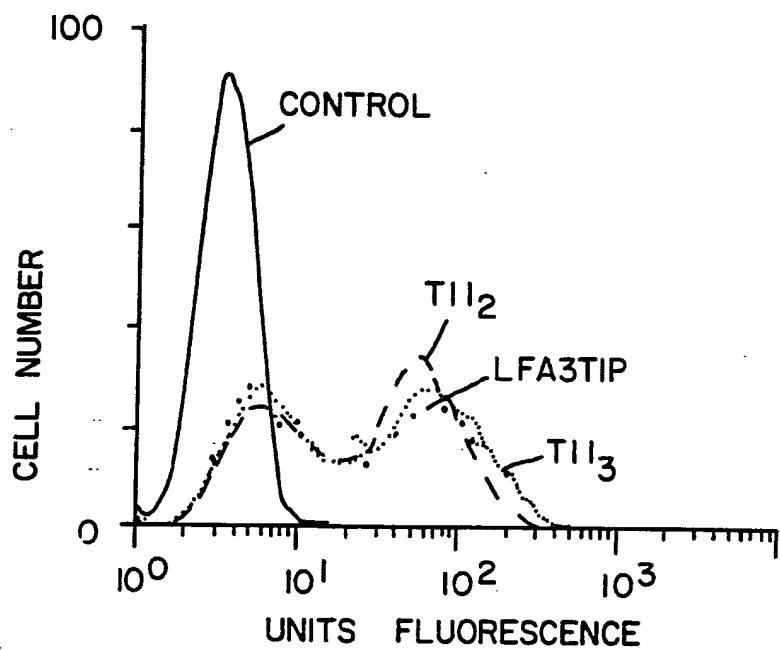
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## FIG. 13



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**FIG. 14A****FIG. 14B****SUBSTITUTE SHEET**

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FIG. 15

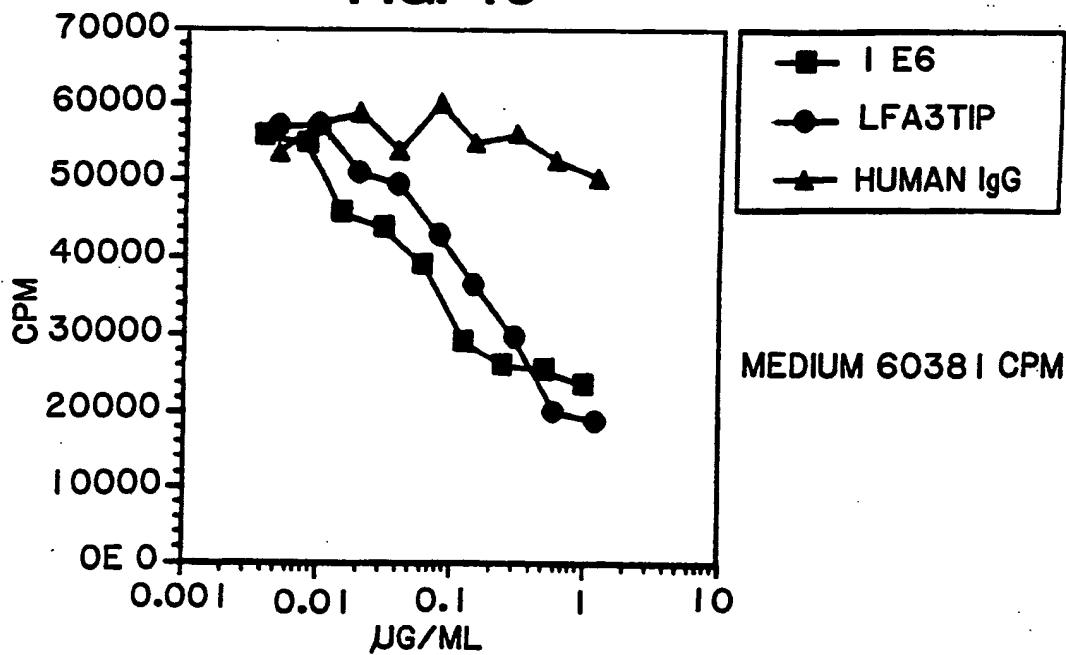
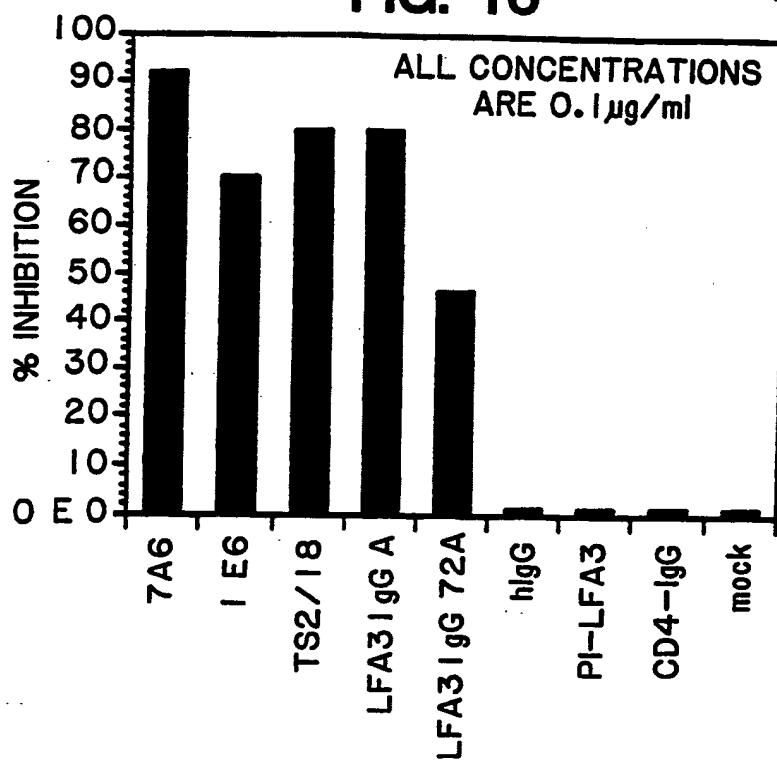


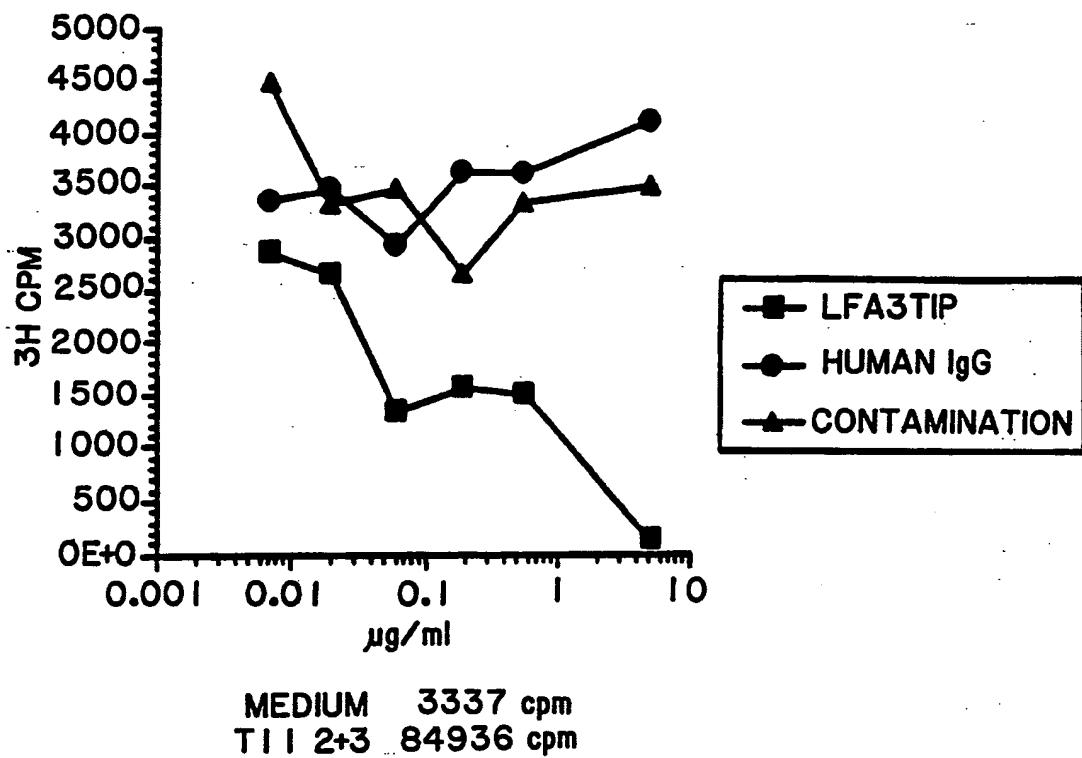
FIG. 16



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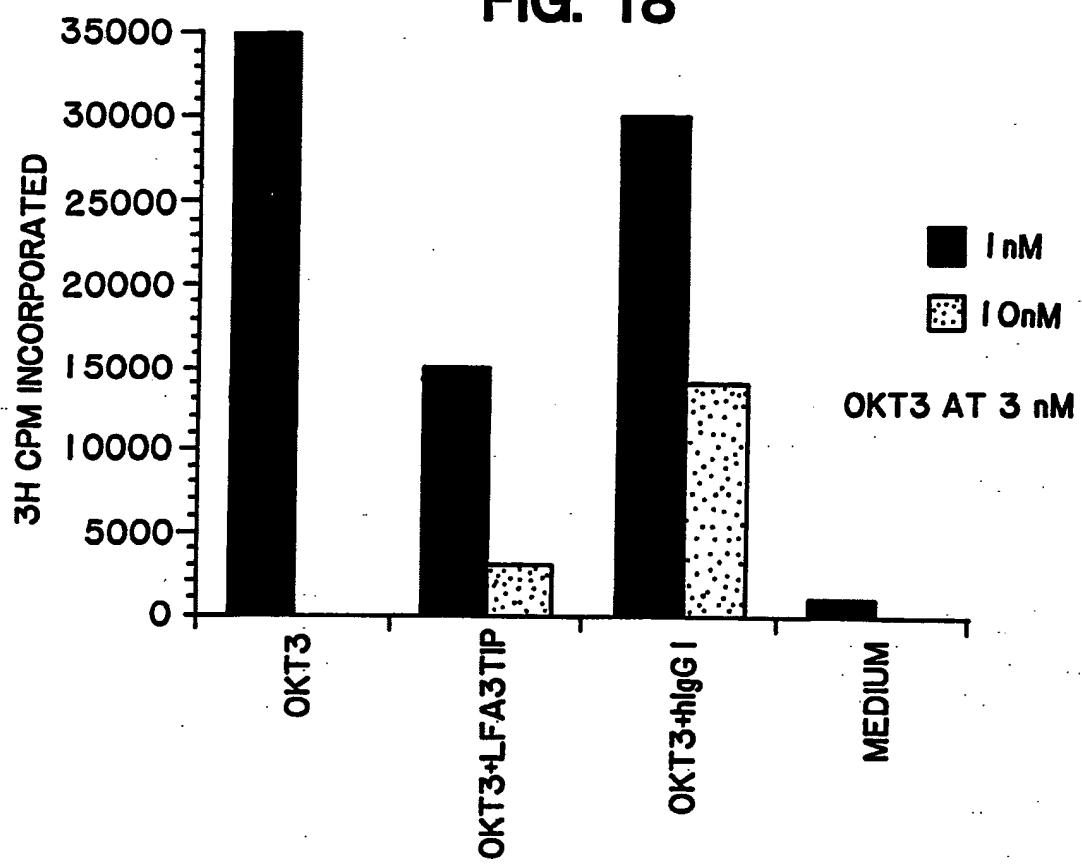
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FIG. 17

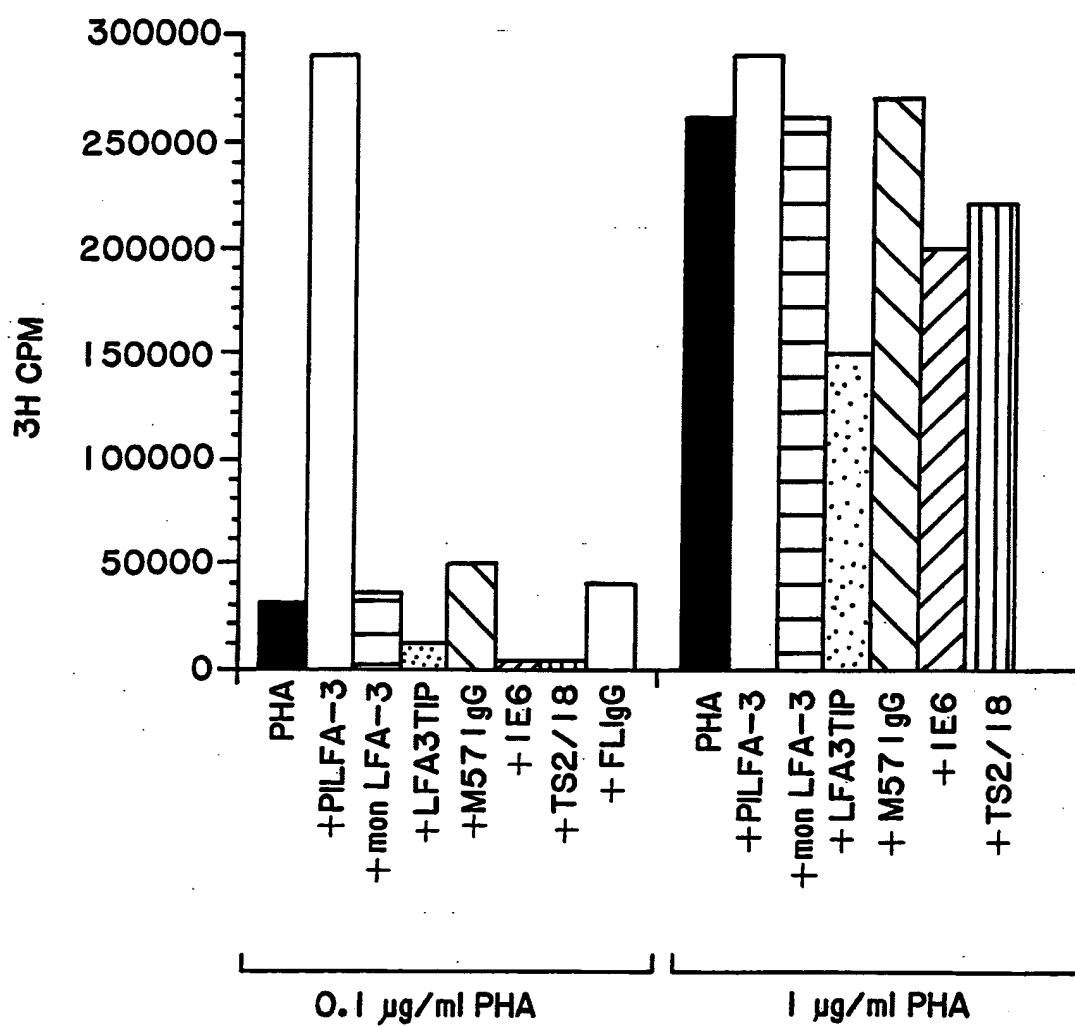


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**FIG. 18****SUBSTITUTE SHEET**

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**FIG. 19****SUBSTITUTE SHEET**

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 92/02050

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (If several classification symbols apply, indicate all) <sup>6</sup>					
According to International Patent Classification (IPC) or to both National Classification and IPC					
Int.C1.5 C 12 N 1/21	C 12 N 15/12 G 01 N 33/564	C 12 N 15/62 G 01 N 33/566	C 07 K 13/00 A 61 K 37/02, 39/395		
<b>II. FIELDS SEARCHED</b>					
Minimum Documentation Searched <sup>7</sup>					
Classification System		Classification Symbols			
Int.C1.5	C 07 K	C 12 N	A 61 K		
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched <sup>8</sup>					
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup></b>					
Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>			Relevant to Claim No. <sup>13</sup>	
X	WO,A,9002181 (BIOGEN, INC. USA) 8 March 1990, see the whole document (cited in the application)			1-9,18- 27	
Y	---			10-17	
Y	EP,A,0314317 (GENENTECH, INC.) 3 May 1989, see abstract; claims; figure 3			10-17	
X	---			1-9,18- 27	
Y	WO,A,8809820 (BIOGEN, INC. USA) 15 December 1988, see the whole document			10-17	
X	---			1-10	
Y	WO,A,9012099 (BIOGEN, INC. USA) 18 October 1990, see the whole document			11-17	
	---			-/-	
<p><sup>10</sup> Special categories of cited documents :          "A" document defining the general state of the art which is not considered to be of particular relevance          "E" earlier document but published on or after the international filing date          "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)          "O" document referring to an oral disclosure, use, exhibition or other means          "P" document published prior to the international filing date but later than the priority date claimed</p> <p><sup>11</sup> T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention          "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step          "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art          "&amp;" document member of the same patent family</p>					
<b>IV. CERTIFICATION</b>					
Date of the Actual Completion of the International Search  08-07-1992	Date of Mailing of this International Search Report  12.09.92				
International Searching Authority  EUROPEAN PATENT OFFICE	Signature of Authorized Officer  S.A. NAUCHE				

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	DNA, vol. 8, no. 10, December 1989, Mary Ann Liebert, Inc., Publishers, (New York, US), X. WANG et al.: "A vector that expresses secreted proteins on the cell surface", pages 753-758 -----	

**ANNEX TO THE INTERNATIONAL SEARCH REPORT**  
**ON INTERNATIONAL PATENT APPLICATION NO. US 9202050**  
**SA 59551**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.  
The members are as contained in the European Patent Office EDP file on 30/07/92  
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A- 9002181	08-03-90	AU-A-	4219489	23-03-90
		EP-A-	0431069	12-06-91
		JP-T-	4500905	20-02-92
-----	-----	-----	-----	-----
EP-A- 0314317	03-05-89	AU-A-	2557188	18-04-89
		EP-A-	0383799	29-08-90
		JP-T-	4502850	28-05-92
-----	-----	-----	-----	-----
WO-A- 8809820	15-12-88	US-A-	4956281	11-09-90
		AU-A-	1955288	04-01-89
		EP-A-	0315683	17-05-89
		JP-T-	2501113	19-04-90
-----	-----	-----	-----	-----
WO-A- 9012099	18-10-90	AU-A-	5428990	05-11-90
		CA-A-	2051694	11-10-90
		EP-A-	0466806	22-01-92
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